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(57) Abstract

This invention relates to enzymes involved in alkaloid, and specifically nicotine, formation in tobacco plants. The invention is based, at least in part, on the nucleotide sequences encoding four variants of putrescine N-methyltransferase (PMT1, PMT2, PMT3, and PMT4), two variants of arginine decarboxylase (ADC1 and ADC2), ornithine decarboxylase (ODC), S-adenosylmethionine synthetase (SAMS), a fragment of NADH dehydrogenase, and a fragment of phosphoribosylanthranilate isomerase. The invention also relates to proteins expressed by these nucleotides, promoter regions of these nucleotides, use of these promoter regions to culture transgenic plant cells and to produce transgenic plants, sense and antisense nucleotides complementary to all or portions of these nucleotide sequences, use of sense and antisense nucleotides to regulate gene expression, and assays using proteins involved in alkaloid formation in tobacco plants.

REGULATION OF GENE EXPRESSION IN TOBACCO FOR MANIPULATION OF PLANT GROWTH AND SECONDARY METABOLISM

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of US Patent Application Ser. No. 60/ 132,919, filed May 6, 1999, now abandoned, which is hereby incorporated by reference in its entirety herein.

FIELD OF THE INVENTION

This invention relates to enzymes involved in alkaloid, and specifically nicotine, formation in tobacco plants. The invention is based, at least in part, on the nucleotide sequences encoding four variants of putrescine N-methyltransferase (PMT1, PMT2, PMT3, and PMT4), two variants of arginine decarboxylase (ADC 1 and ADC2), ornithine decarboxylase (ODC), S-adenosylmethionine synthetase (SAMS), a fragment of NADH dehydrogenase, and a fragment of phosphoribosylanthranilate isomerase. The invention also relates to proteins expressed by these nucleotides, promoter regions of these nucleotides, use of these promoter regions to culture transgenic plant cells and to produce transgenic plants, sense and antisense nucleotides complementary to all or portions of these nucleotide sequences, use of sense and antisense nucleotides to regulate gene expression, and assays using proteins involved in alkaloid formation in tobacco plants.

BACKGROUND OF THE INVENTION

I. Alkaloid Formation

Alkaloids are one of the most diverse groups of secondary compounds found in plants and they are the product of a complex biosynthesis pathway (Hashimoto and Yamada, 1994; Chou and Kutchan, 1998; Waterman, 1998). Why plants accumulate these compounds and in so many different forms is not known. Moreover, for many alkaloids, the exact site of synthesis and the factors that control their intercellular distribution and accumulation remain to be determined (Hashimoto and Yamada, 1994; Kutchan, 1995; Chou and Kutchan, 1998).

Nicotine is the most abundant alkaloid present in cultivated tobacco. Nicotine is formed primarily in the roots of the tobacco plant and subsequently is transported to the leaves, where it is stored (Tso, Physiology and Biochemistry of Tobacco Plants, pp. 233-34, Dowden, Hutchinson & Ross, Stroudsburg, Pa. (1972)).

The synthesis and accumulation of nicotine and other tobacco alkaloids are known to be controlled by various developmental, environmental, and chemical cues. Changes in phytohormone

(e.g., auxin, cytokinin) levels and/or ratios as a consequence of developmental age (Hashimoto and Yamada, 1994; Kutchan, 1995) or by direct manipulation of plant cell culture conditions have been shown to affect the synthesis and accumulation of nicotine and various tobacco alkaloids (Hashimoto and Yamada, 1994; Hibi *et al.*, 1994; Eilbert, 1998). Various abiotic factors (wounding, drought stress, pH imbalance, etc.) [Hashimoto and Yamada, 1994; Kutchan, 1998; Waterman, 1998) 1, 2, 4], as well as biotic factors, such as herbivory, insect feeding, and attack by various microbial and fungal pathogens, are known elicit increased production of nicotine and other alkaloids in the leaves of wild and cultivated tobacco species (Baldwin, 1989; Saito and Murakoishi, 1998; Baldwin and Prestin, 1999). In addition, the commercial practice of topping (i.e., removal of flowering head and young leaves at the upper portions of the plant), results in increases in nicotine and the amount and complexity total alkaloids present in the leaves of *Nicotiana tabacum* (Hashimoto and Yamada, 1994; Hibi *et al.*, 1994). The factors controlling the topping-induced increase in alkaloid biosynthesis are not known, but likely involve a complex physiological response in the plant as a result of altered phytohormones and wound induced signaling (Akehurst, 1981; Hibi *et al.*, 1994; Kutchan, 1998). In this regard, considerable evidence now exists indicating that a jasmonic acid (JA)- mediated signal transduction pathway may play a role in regulation of gene expression contributing to this increase in alkaloid biosynthesis (Baldwin *et al.*, 1994, 1996,1997; Ohnmeiss *et al.*, 1997; Imanishi *et al.*, 1998*a*, 1998*b*).

The nicotine molecule is comprised of two heterocyclic rings, a pyridine moiety and a pyrrolidine moiety, each of which is derived from a separate biochemical pathway. The pyridine moiety of nicotine is derived from nicotinic acid. The pyrrolidine moiety of nicotine is provided through a pathway leading from putrescine to N-methylputrescine and then to N-methylpyrroline. (Goodwin and Mercer, Introduction to Plant Biochemistry, pp. 488-91, Pergamon Press, New York, (1983)).

Putrescine is formed in plants by one of two pathways (Chattopadhyay and Ghosh, 1998). It can be synthesized directly from ornithine, in a reaction catalyzed by the enzyme ornithine decarboxylase (ODC, EC 4.1.1.17), or formed indirectly from arginine in a reaction sequence initiated by arginine decarboxylase (ADC, EC 4.1.1.19). Putrescine formed by the ADC and/or ODC pathway serves as precursor in the synthesis of the higher polyamines, spermine and spermidine, catalyzed by the enzymes spermine synthase and spermidine synthase, respectively, or it is converted to N-methylputrescine by the action of putrescine N-methyltransferase (PMT), the first committed step in nicotine biosynthesis (Hashimoto and Yamada, 1994; Kutchan, 1995; Chattopadhyay and Ghosh, 1998). N-methyl putrescine is oxidized by a diamine oxidase and cyclized to form the 1-methyl-Δ^1-pyrrolium cation, which is condensed with nicotinic acid or its derivative to form nicotine

(Hashimoto and Yamada, 1994).

Putrescene is a precursor for N-methylputrescine, which then forms N-methylpyrroline. Conversion of putrescine to N-methylputrescine is catalyzed by the enzyme putrescine N-methyltransferase ("PMT"), with S-adenosylmethionine serving as the methyl group donor. PMT appears to be the rate-limiting enzyme in the pathway supplying N-methylpyrroline for nicotine synthesis in tobacco (Feth et al., "Regulation in Tobacco Callus of Enzyme Activities of the Nicotine Pathway", Planta, 168, pp. 402-07 (1986); Wagner et al., "The Regulation of Enzyme Activities of the Nicotine Pathway in Tobacco", Physiol. Plant., 68, pp. 667-72 (1986)).

II. TRANSGENIC PLANTS

The methods of nicotine formation in tobacco and the genes involved have been studied both to better understand differential gene expression during tobacco growth and development, and also to discover tools useful for creating transgenic plants. For example, the regulatory sequences that modify protein expression in tobacco may be useful in creating transgenic tobacco or other transgenic plants.

It has already been demonstrated that tissues of many plant species may be transformed by exogenous, typically chimeric, genes which are effective to stably transform cells of the tissues. For several species, tissues transformed in this fashion may be regenerated to give rise to whole transqenic or genetically engineered plants. The engineered traits introduced into the transgenic plants by these techniques have proven to be stable and have also proven to be transmissible through normal Mendellian inheritance to the progeny of the regenerated plants. One such desirable trait is the production in the plant cells of desired gene products in vivo in the cells of the transqenic plants. For a chimeric gene to be effective, the foreign DNA sequence containing a coding region should be flanked by appropriate promotion and control regions. Commonly used plant cell transcription promoters include the nopaline synthase promoter from the T-DNA of A. tumefaciens and the 35S promoter from the cauliflower mosaic virus.

In order for the newly inserted chimeric gene to express the protein for which it codes in the plant cell, the proper regulatory signals must be present and in the proper location with respect to the gene. These regulatory signals include a promoter region, a 5' non-translated leader sequence and a 3' polyadenylation sequence. A promoter is a DNA sequence that directs the cellular machinery of a plant to produce RNA from the contiguous structural coding sequence downstream (3') to the promoter. The promoter region influences the rate at which the RNA product of the gene and resultant protein product of the gene is made. The 3' polyadenylation signal is a non-translated region that functions in

the plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA to enable the mRNA to be transported to the cytoplasm and to stabilize the mRNA for subsequent translation of the RNA to produce protein.

Other plant cell transformation techniques are directed toward the direct insertion of DNA into the cytoplasm of plant cells from which it is taken up, by an uncharacterized mechanism, into the genome of the plant. One such technique is electroporation, in which electric shock causes disruption of the cellular membranes of individual plant cells. Plant protoplasts in aqueous solution when subject to electroporation will uptake DNA from the surrounding medium. Another technique involves the physical acceleration of DNA, coated onto small inert particles, either into reqenerable plant tissues or into plant germline cells.

The availability of cloned nucleic acid sequences encoding an enzyme involved in alkaloid synthesis allows for the potential manipulation of alkaloid contents. Furthermore, the availability of promoters useful for expressing genes in plants allows for the creation of chimeric molecules and transgenic plants, which in turn result in possible native plant production of desirable proteins.

Previously reported work discloses cloning nucleotides encoding proteins involved in the biosynthesis of nicotine, and isolating such proteins. Approximately twenty or more cDNAs and/or genomic DNA fragments encoding different enzymes involved with alkaloid formation have been isolated (Chattopadhyay and Ghosh, 1998). For example, successful cloning of partial or full-length cDNA encoding ODC activity from tobacco was disclosed by (Malik *et al.*, *J. Plant Biochem. &Biotech. 5:*109-112 (1996)). Also, a relatively crude preparation of PMT (30-fold purification) has been subjected to limited characterization (Mizusaki et al., "Phytochemical Studies on Tobacco Alkaloids XIV. The Occurrence and Properties of Putrescine N-Methyltransferase in Tobacco Plants", Plant Cell Physiol., 12, pp. 633-40 (1971)). A process for purifying PMT is disclosed in US Patent No. 5,369,023, "Method of purifying putrescine n-methyltransferase from tobacco plant extract with an anion exchange medium", hereby incorporated by reference in its entirety herein. Several laboratories have reported the cloning of partial or full-length cDNAs encoding ADC (Bell and Malmberg ,1990; Rostogi et al., 1993; Perez-Amador et al., 1995; Nam et al., 1997; Watson and Malmberg, 1996). Comparisons of the amino acid sequences of ADC from various plants revealed a high degree of conservation among the various proteins, as well as homology to ODC (Malmberg et al., 1998).

It is an object of the present invention to characterize the nucleotide and amino acid sequences of enzymes involved in the biosynthesis of nicotine in tobacco. It is also an object of the present invention to provide plant promoter regions that are capable of conferring high levels of transcription in rapidly dividing cells of transformed plants when coupled with a heterologous coding

sequence in a chimeric gene. Further, the invention is directed to chimeric genes incorporating such promoter regions, stable transfection of plants with these chimeric genes, and the plants and cells that are transfected, as well as seeds of such transfected plants. It is a further object to characterize sense and antisense nucleotides capable of regulating expression of genes encoding for enzymes involved in the biosynthesis of alkaloids.

SUMMARY OF THE INVENTION

Proteins involved in the biosynthesis of nicotine in tobacco *N. tabacum* are the subject of this invention. More specifically, the invention concerns four variants of putrescine N-methyltransferase (PMT1, PMT2, PMT3, and PMT4), two variants of arginine decarboxylase (ADC 1 and ADC2), ornithine decarboxylase (ODC), S-adenosylmethionine synthetase (SAMS), NADH dehydrogenase, and phosphoribosylanthranilate isomerase.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Genomic DNA gel blot analysis of the PMT gene family in *N. tabacum* cv. Xanthi. Total genomic DNA (30 µg) was digested with *Kpn*I, *Eco*RI, or *Eco*RI and *Kpn*I, separated by agarose gel electrophoresis, and transferred to nylon membranes. The membrane was hybridized with a ^{32}P-labeled antisense strand probe covering the complete coding region of the *NtPMT1a* cDNA. Identity of the hybridizing bands as determined by comparison to phage DNA digests is indicated. Molecular weights are given in kb. Note that *Kpn*I shifts only the *NtPMT1b* band in the gel blot because this restriction site is present ony in Exon 1 of *NtPMT1b* and not *NtPMT1a*.

Figure 2. Amino acid sequence alignment of *N. tabacum* PMTs. Shown is a PILEUP alignment of the predicted amino acid sequences of the various tobacco PMTs. Amino acid residues that differing among the PMTs are shaded. NtPMT1a, NtPMT2, NtPMT3, and NtPMT4 refer to the deduced amino acid sequences of the PMTs encoded by the *NtPMT1a*, *NtPMT2*, *NtPMT3*, and *NtPMT4* genes, respectively, isolated from *N. tabacum* cv. Xanthi genomic DNA; cNtPMT1a is the predicted amino acid sequence of the A411 cDNA (Accession No. D28506) isolated from *N. tabacum* cv. Burley 21 by Hibi *et al.* (1994). The location of the exon-intron boundaries are indicated by the dark vertical line. The nucleotide sequences for *NtPMT1a*, *NtPMT2*, *NtPMT3*, and *NtPMT4* appear in GenBank under the accession numbers AF126810, AF126809, AF126811, and AF126812, respectively

Figure 3. Polyacrylamide gel electrophoresis analysis of PCR amplified genomic DNA fragments

encoding Exon 1 of PMT from various species of *Nicotiana*. PCR amplification was carried out as described in the Materials and Methods using Exon 1-specific primers 1 and 2 and total genomic DNA isolated from *N. tabacum*, *N. otophora*, and *N. tomentosiformis*. The amplification products were separated by electrophoresis on 6.5% polyacrylamide gels, the gels fixed, and subject to autoradiography. The amplification products isolated from *N. tabacum* cv. Burley 21 and *N. tabacum* cv. Xanthi were identical and only the amplication products from the reactions with *N. tabacum* cv. Burley 21 DNA are shown. Standards were generated in identical reaction conditions primed with plasmid DNA encoding the various *PMT* genes isolated in this study.

Figure 4. Nucleotide sequence alignment of the 5'-flanking regions of the *N. tabacum PMT* genes. Shown is a PILEUP alignment of the nucleotide sequences upstream of the initiating methionine (MET) codon of the four *PMT* genes isolated from *N. tabacum* cv. Xanthi. The proposed start site for transcription of the *NtPMT1a* gene is indicated by the +1 above the sequences. The TATA-box and CCAAT-box motifs are boxed. Potential transcriptional regulatory elements identified by MOTIF search programs are also boxed and indicated by the following abbreviations:. PAL: palindromic sequences; G-Box: G-Box homologous sequences; MRE: metal-responsive element homolog. Nucleotides identical in three or more sequences are shaded. The polyguanine-rich region is underlined. Numbering is indicated to the right and is relative to the proposed start site of each gene.

Figure 5. RNA gel blot analysis of *PMT* transcript levels in various tissues. Total RNA was isolated from various tissues of mature *N. tabacum* cv. Burley 21 and analyzed by gel blot analysis using a ^{32}P-labeled *NtPMT1a* cDNA coding region (Exons 2 to 8) probe capable of detecting all *PMT* transcripts.

A. *PMT* transcript levels in various tobacco plant tissues and/or organs.

B. Induction of *PMT* expression in tobacco roots following topping. Abbreviations: HP, wild-type (*Nic1Nic2*) Burley 21; LP, low alkaloid (*nic1nic2*) mutant. The β-subunit of mitochondrial ATPase (β-ATPase) served as a control.

Figure 6. Semi-quantitative RT-PCR analysis of *PMT* gene expression in roots of tobacco plant before and after topping.

A. Shown is relative abundance of the individual *PMT* gene transcripts before and after topping. RT-PCR was carried out as described in the Material and methods using Exon 1 specific primers. Messenger RNA was amplified from total RNA isolated from the roots of wild-type (HP,

Nic1Nic2) Burley 21 and low alkaloid (LP, *nic1nic2*) Burley 21 tobacco plants. Far right lane represents size standards for the genes isolated by PCR amplification from plasmid DNA. The β-subunit of mitochondrial ATPase (β-ATPase) served as a control.

B. Bar graphs showing relative expression of the individual PMT genes following topping in both HP and LP tobacco roots. Abbreviations: HP, wild-type (*Nic1Nic2*) Burley 21; LP, low alkaloid (*nic1nic2*) mutant.

Figure 7. The nucleotide and predicted amino acid sequences of the transcribed portions of the *N. tabacum* cv Xanthi NtADC1 and NtADC2 genes. Shown are the complete nucleotide and predicted amino acid sequence of the *N. tabacum* cv Xanthi NtADC1 gene and where it differs from the NtADC2 gene sequence. The dots indicate nucleotide sequence identity and the stars indicate amino acid sequence identity. The proposed polyadenylation signal is underlined. The sequences terminate at the point of polyadenylation found in the full length cDNA (Wang, 1999; AF127239).The complete nucleotide sequences for the *N. tabacum* cv Xanthi NtADC1 (AF127240) and NtADC2 (AF127241) including the 5' and 3' flanking sequences appear in Genbank.

Fig. 8. Comparison of the predicted amino acid sequences of arginine decarboxylases (ADCs) from various species. Shown is a PILEUP alignment of the predicted amino acid sequence of the *N. tabacum* cv Xanthi NtADC1 gene (AF127240) aligned to the predicted ADC protein sequences from *N. sylvestris* (AB12873), *Arabidopsis thaliana* (AF009647), *Avena sativa* (oat) (X56802), *Lycopersicon esculentum* (tomato) (L16582) and *Escherichia coli* (M31770). Amino acid residues conserved among the various ADC are shaded.

Fig. 9. Gel blot analysis of *ADC* transcript levels in the roots of wild-type and low alkaloid mutant Burley 21 tobacco before and after topping. Total RNA was isolated from the roots of mature wild-type and low alkaloid mutant *N. tabacum* cv. Burley 21 and analyzed by gel blot analysis using [α-^{32}P]-dCTP labeled probes recognizing the coding region of ADC or the β-subunit of tobacco mitochondrial ATP synthase (Boutry and Chua, 1985). Quantitation was carried out by phosphorimaging using a Molecular Dynamics PhosphorImager. Values were normalized relative to the intensities of the *atp2* control band in each lane. The experiment was conducted twice with different total RNA samples.

Fig. 10. Differential expression of NtADC-1 and NtADC-2 in various tissues of tobacco. Expression of the NtADC-1 and NtADC-2 genes was analyzed using semi-quantitative RT-PCR and gene specific primers capable of discriminating between transcripts arising from the two genes. Panel A shows control reactions demonstrating primer specificity in the PCR reactions using plasmids containing the NtADC-1 and NtADC-2 coding sequences. The numbers above the lane refer to the specific primer combinations as described in the Materia and methods. Panel B shows the results of RT-PCR reactions using first strand cDNA synthesized from total RNA extracted from either root, leaf, or flowers. As a internal control, primers specific for the *atp2* gene transcript were include in the amplification reactions. All reactions were carried out within the linear range of template amplification as determined by varying template amount, amplification time, and temperature as described in Riechers and Timko (1999).

Fig. 11. Genomic DNA gel blot analysis of the ODC gene family in *N. tabacum*. Total genomic DNA (30 μg) was digested with *Eco*RI or *Hind*III, fractionated by agarose gel electrophoresis, transferred to nylon membranes and hybridized with an α-^{32}P-dCTP labeled probe encoding full-length ODC cDNA as described in the Materials. The mobility of molecular weights standards are given to the right of the figure in kilobases (kb).

Fig 12. Comparison of the nucleotide and predicted amino acid sequences of the *NtODC-1* and *NtODC-2* genes. Shown are the nucleotide and predicted amino acid sequences of the *NtODC-1* (AF233850) and *NtODC-2* (AF233849) genes. In the figure, the complete amino acid sequence of the pODC2 is given and the pODC1 sequence is given only where it differs. The start site of transcription is designated as +1 and the poly(A) addition site is indicated by the arrow. Within the relevant regions of homology, nucleotide differences between the *NtODC-1* and *NtODC-2* genes are in bold lettering. The proposed TATA-box, and polyadenylation signal are shaded.

Fig. 13. Protein sequences alignment of ornithine decarboxylases (ODCs) from various species. Shown is a PILEUP alignment of the predicted amino acid sequences of the *N. tabacum* cv. Xanthi pODC2 protein (AF233849) with the ODCs from *N. tabacum* cv. SC58 (Y10472) and cv. BY-2 (ABO31066), *Lycopersicon esculentum* (tomato) (AF030292), *Datura stramonium* (jimsonweed) (X87847), *Saccharomyces cerevisiae* (NP_012737), and humans (*Homo sapiens*; AAA59966). Amino acid residues conserved among the various ODCs are shaded.

Fig. 14. Gel blot analysis of *ODC* transcript levels in various tissues of mature tobacco plants and in the roots before and after topping. Total RNA was isolated from various tissues of mature *N. tabacum* cv. Burley 21 and analyzed by gel blot analysis using an α-^{32}P-dCTP labeled coding region probes for ODC. (A) Transcript levels in various organs of wild-type tobacco: R, root: S, stem ; L, leaf ; SE, sepal; PE, petal; O, ovary; S, stamen; and AN, anther. (B) Transcript levels in roots of Burley 21 tobacco plants before and after topping. RNA gel blot analysis of the tissues-specific distribution and post-topping expression of transcripts encoding ODC in tobacco. As a control, the blots were also probed with radioactively labeled probes encoding the alkaloid biosynthesis enzyme putrescine N-methyltransferase (PMT) and a root specific β-glucosidase (TBG-1).

DETAILED DESCRIPTION OF THE INVENTION

Nucleic acid sequences have been isolated from tobacco that encode important enzymes in nicotine and total alkaloid formation, including PMT1, PMT2, PMT3, PMT4, ADC1, ADC2, ODC, and SAMS. Also identified are cDNA fragments encoding partial segments of NADH dehydrogenase and phosphoribosilanthronilate isomerase. Also identified are promoter regions for the nucleotides encoding PMT1, PMT2, PMT3, PMT4, and ADC2. All of these nucleic acids are isolated from *Nicotiana tabacum* L.

"Promoter" and "promoter region" are terms used interchangeably herein to refer to a DNA sequence that regulates expression of a selected DNA sequence operably linked to the promoter, and which effects expression of the selected DNA sequence in cells. The term also encompasses the 5'untranslated region that may be transcribed into mRNA but is not translated.

"Protein", "polypeptide", and "peptide" are used interchangeably herein when referring to a gene product.

In one aspect, the invention features isolated nucleic acid molecules encoding for PMT1, PMT2, PMT3, PMT4, ADC1, ADC2, ODC, and SAMS, a fragment of NADH dehydrogenase and a fragment of phosphoribosilanthronilate isomerase. The disclosed molecules can be non-coding (e.g. probe, antisense or ribozyme molecules) or can code for a functional enzyme. In one embodiment, the nucleic acid molecules can hybridize to the nucleic acid sequences encoding for PMT1, PMT2, PMT3, PMT4, ADC1, ADC2, ODC, SAMS, a fragment of NADH dehydrogenase, or a fragment of phosphoribosilanthronilate isomerase or to the complements of these nucleic acid sequences. In a preferred embodiment, the hybridization is conducted under mildly stringent or stringent conditions.

In further embodiments, the nucleic acid molecule is at least 50%, 60%, 70%, 80% and more preferably at least 90% or 95% homologous in sequence to the nucleic acid sequences encoding for PMT1, PMT2, PMT3, PMT4, ADC1, ADC2, ODC, SAMS, a fragment of NADH dehydrogenase, or

a fragment of phosphoribosilanthronilate isomerase or to the complements of these nucleic acid sequences. In another embodiment, the nucleic acid encodes a polypeptide that is at least 50%, 60%, 70%, 80% and more preferably at least 90% or 95% similar in sequence to the amino acid sequence of PMT1, PMT2, PMT3, PMT4, ADC1, ADC2, ODC, SAMS, the fragment disclosed herein of NADH dehydrogenase, or the fragment of phosphoribosilanthronilate isomerase disclosed herein.

In another embodiment, the invention features isolated polypeptides, preferably substantially pure preparations, encoded for by the nucleic acid sequences of the invention. Particularly preferred are those polypeptides encoded for by the nucleic acid sequences identified by (SEQ. ID. NO. 2), (SEQ. ID. NO. 5), (SEQ. ID. NO. 8), (SEQ. ID. NO. 11), (SEQ. ID. NO. 13), (SEQ. ID. NO. 15), (SEQ. ID. NO. 18), (SEQ. ID. NO. 21), (SEQ. ID. NO. 23), (SEQ. ID. NO. 25) or (SEQ. ID. NO. 26) or comprising a nucleotide sequence encoding the amino acid sequence encoded by (SEQ ID NO. 3), (SEQ. ID. NO. 6), (SEQ ID. NO. 9), (SEQ. ID. NO. 12), (SEQ. ID. NO. 14), (SEQ. ID. NO. 16), (SEQ. ID. NO. 19), (SEQ. ID. NO. 22) or (SEQ. ID. NO. 24). In particularly preferred embodiments, the subject polypeptides can aid in regulating the production of alkaloids, particularly nicotine, in plants. In one embodiment, the polypeptide is identical to or similar to the protein represented by the amino acid sequences of (SEQ ID NO. 3), (SEQ. ID. NO. 6), (SEQ ID. NO. 9), (SEQ. ID. NO. 12), (SEQ. ID. NO. 14), (SEQ. ID. NO. 16), (SEQ. ID. NO. 19), (SEQ. ID. NO. 22) or (SEQ. ID. NO. 24). In a preferred embodiment, the polypeptide is encoded by a nucleic acid that hybridizes with a nucleic acid represented in.

The polypeptides of the present invention can comprise full length proteins, such as represented by (SEQ ID NO. 3), (SEQ. ID. NO. 6), (SEQ ID. NO. 9), (SEQ. ID. NO. 12), (SEQ. ID. NO. 14), (SEQ. ID. NO. 16), (SEQ. ID. NO. 19), (SEQ. ID. NO. 22) and (SEQ. ID. NO. 24), or can comprise one or more fragments corresponding to one or more particular motifs/domains, or to arbitrary sizes, e.g., at least 5, 10, 25, 50, 100, 150, or 200 amino acids in length.

Another aspect of the invention features chimeric genes comprised of a promoter for the genes for PMT2, PMT1, PMT3, PMT4, or ADC2. Yet another aspect of the invention features chimeric genes or chimeric molecules comprised respectively of the functional gene encoding for or the protein PMT1, PMT2, PMT3, PMT4, ADC1, ADC2, ODC, SAMS, NADH dehydrogenase and/or phosphoribosilanthronilate isomerase.

The invention also concerns isolated and purified promoter regions for tobacco Beta-glucosidase and their use in chimeric genes or chimeric molecules.

Another aspect of the invention involves vectors capable of transporting another nucleic acid to which a vector has been linked. Preferably, the vectors comprise the nucleic acid sequences of the invention or their complements.

The invention also features transgenic plants and their seeds that include (and preferably express) a heterologous form of PMT1, PMT2, PMT3, PMT4, ADC1, ADC2, ODC, SAMS, NADH dehydrogenase and/or phosphoribosilanthronilate isomerase. The present invention also encompasses transgenic plants that contain in their genome a chimeric gene construction incorporating the nucleic acid encoding PMT1, PMT2, PMT3, PMT4, ADC1, ADC2, ODC, SAMS, NADH dehydrogenase and/or phosphoribosilanthronilate isomerase. Such transgenic plants and their seeds may be useful to natively produce enhanced quantities of desirable exogenous proteins, such as compounds useful for pharmaceutical purposes, or proteins that provide herbicide resistance.

Another feature of the invention is the use as probes of the DNA sequences disclosed herein or oligonucleotide fragments thereof. Probes may be useful to obtain additional gene family members or locate homologous genes in tobacco or other plant species. Copies of related genes can be obtained from existing genomic libraries or the genomic libraries can be constructed. In one embodiment, an isolated DNA sequence comprising about a fifteen to about a twenty-five base pair oligonucleotide sequence identical to any consecutive about fifteen to about twenty-five base pair sequence found in the sequences of the invention is used as a probe.

Another feature is use of the polypeptides of the invention in an assay, such as an assay to identify modulators of enzyme activity in plants.

Other features and advantages of the invention will be apparent to those of skill in the art.

The nucleotide and amino acid sequences of the invention are disclosed herein in the Sequence Listing, text, and the figures. Specific sequences of the invention are provided in the attached Sequence Listing and can be understood to represent promoters, nucleic acids, and proteins respectively relating to the following proteins: PMT2 (SEQ. ID. NOS. 1, 2, and 3); PMT1 (SEQ. ID. NOS. 4, 5, and 6); PMT3 (SEQ. ID. NOS. 7, 8, and 9); PMT4 (SEQ. ID. NOS. 10, 11, and 12); SAMS (SEQ. ID. NOS. 13 and 14); ODC (SEQ. ID. NOS. 15 and 16); ADC1 (SEQ. ID. NOS. 17, 18, and 19); ADC2 (SEQ. ID. NOS. 20, 21, and 22); ADC1 mRNA (SEQ. ID. NOS. 23 and 24); NADH dehydrongenase (SEQ. ID. NO. 25); and PAI (SEQ. ID. NO. 26). If only two sequence identifiers are provided for a protein, those sequences represent the nucleic acid and the protein respectively. If three identifiers are provided, the identifiers represent promoter, genomic or cDNA nucleic acid, and peptide sequences, respectively. If only one identifier is provided, it represents a DNA fragment coding for the protein or portions of it.

For other reference, the sequences may be found at the following records in GenBank at the following Accession Numbers, which records are hereby incorporated in their entirety herein: AF126810 (NtPMT1); AF126809 (NtPMT2); AF126811 (NtPMT3); AF126812 (NtPMT4), AF176908 (NtomPMT)(Nicotiana tomentosiformis); AF76909 (NotoPMT)(Nicotiana otophora);

AF127239 (ADC); AF127240 (ADC1); AF127241 (ADC2); AF127242 (ODC); AF233849 (ODC2); AF233850 (ODC1); and AF127243 (SAMS).

The following experimental discussion is presented to better illustrate the invention.

I. PMT

The present invention features the characterization of four members of the nuclear gene family encoding PMT in tobacco *N. tabacum*. The nucleic acid sequences encoding PMT and the amino acid sequences for the proteins are reported herein and can also be found in the DDBJ, EMBL, and GenBank Nucleotide Sequence Databases under the accession numbers for *NtPMT1a*, *NtPMT2*, *NtPMT3*, and *NtPMT4* at AF126810, AF126809, AF126811, and AF126812, respectively. The complete coding region and immediate 5'- and 3'- flanking regions are characterized.

As the discussion below shows, all four PMT genes present in the *N. tabacum* genome are expressed in the roots of wild-type plants and differentially regulated in tobacco lines expressing either high or low total alkaloid contents.

Materials and Methods

Plant materials

Seeds of *N. sylvestris*, *N. otophora*, and *N. tomentosiformis* were obtained from the USDA-ARS national tobacco germplasm collection (Oxford, NC). *N. tabacum* cv. Burley 21 and *N. tabacum* cv. Xanthi seeds were kindly provided by Glenn Collins, University of Kentucky. Tobacco plants used for DNA isolation were grown in a soil:vermiculite mixture in the greenhouse under natural lighting conditions. Plants used for RNA extraction were grown in Moltan Plus (Moltan Co., Middleton, TN).

Gel blot analysis of genomic DNA

Young leaves were collected from greenhouse grown tobacco (*N. tabacum* cv. Xanthi) plants and total genomic DNA was isolated from freshly-harvested tissues using a modification of the CTAB extraction method (Dellaporta *et al.*, 1983). Approximately 30 µg of total DNA was digested with *Eco*RI, *Kpn*I, or *Eco*RI and *Kpn*I and the digestion products separated by electrophoresis through a 0.75% agarose gel. Restricted and size-fractionated DNA was denatured and transferred to Nytran+ nylon membranes (Schleicher and Schuell, Keene, NH) by capillary blotting in 0.4N NaOH overnight. Membranes were prehybridized in 0.25M Na_2HPO_4 (pH 7.4), 7% SDS, 1 mM Na_2EDTA

for at least 2 hr, then hybridized overnight at 65°C in the same buffer with 2-3 x 10^6 cpm/mL of a ^{32}P-labeled single-stranded probe (antisense DNA strand). The probe was prepared by the method of Bednarczuk *et al.* (1991) using a primer (Table 1, primer 4) designed from the 3' end of the *NtPMT1a* coding region (Exon 8) and the full-length coding region of the *NtPMT1a* cDNA as template. The *NtPMT1a* cDNA was generated by RT-PCR using synthetic oligonucleotide primers based on the N- and C-terminal sequences of the A411 cDNA reported by Hibi *et al.* (1994) and RNA template isolated from *N. tabacum* cv. Burley 21 roots. Membranes were washed at a final stringency of 0.1 x SSC, 0.1% SDS at 65°C. Hybridizing bands were visualized by autoradiography and/or imaged using a Molecular Dynamics PhosphorImager (Model 445 SI, Sunnyvale, CA).

Genomic library construction and phage isolation

A library of *N. tabacum* cv. Xanthi genomic DNA fragments constructed in EMBL3 was purchased from Clontech (Palo Alto, CA) and a total of 1.1 x 10^6 recombinant phage were screened by plaque hybridization using random-primed ^{32}P-labeled *NtPMT1a* cDNA as probe (Sambrook *et al.*, 1989). Prehybridization, hybridization, and washing conditions were as described above. Positive hybridizing phage were plaque purified by subsequent rounds of rescreening and DNA was prepared from 18 independently isolated phage. The phage DNA was characterized by restriction analysis and DNA gel blot analysis and fragments containing the sequences encoding PMT were subcloned into pBluescript KS vectors for further analysis.

Comparison of the hybridizing fragments present in the 18 recombinant phage to the hybridization pattern obtained by genomic DNA blot analysis indicated that only three of the *PMT* genes suspected to be present in the *N. tabacum* genome were recovered from the library screen. To obtain sequences encoding *NtPMT1a*, a subgenomic library was constructed from *N. tabacum* cv. Xanthi DNA. The library consisted of gel-purified 2.5-3.5 kb *Eco*RI fragments ligated into λ_ZAP II vector arms and packaged using Gigapack III Gold packaging extracts according to the manufacturer's instructions (Stratagene, La Jolla, CA). The primary library was amplified once in *E. coli* XL1-Blue MRF' strain (Stratagene) and screened as described above, except that a random-primed ^{32}P-labeled *NtPMT1a* cDNA Exon 1-specific probe was used (Table 1). Exon 1 had previously been amplified by PCR using primers 1 and 2 (Table 1) and the *NtPMT1a* cDNA as template. The recombinant phage that hybridized with the probe was isolated from the sublibrary by two more rounds of plaque purification, and the pBluescript phagemid containing the approximate 3.1 kb *Eco*RI genomic fragment with the *NtPMT1a* gene was excised from the λ_ZAP II phage vector using the *in vivo* excision protocol described by Stratagene.

DNA sequence analysis

Unless otherwise noted, DNA sequencing was performed with double-stranded plasmid DNA templates using fluorescent dye terminator technology (dRhodamine Terminator Cycle Sequencing Ready Reaction kit) on an ABI 310 DNA sequencer (Perkin-Elmer Applied Biosystems). For analysis of PCR products, following electrophoretic separation of amplification reaction products, the bands of interest were excised from the polyacrylamide gels, the DNA extracted using the Quiagen Gel Extraction Kit, and the recovered DNA used as sequencing template. Sequencing was performed using AmpliTaq DNA polymerase and fluorescent dye terminator technology (as described above) and primers 1 and 2 (Table 1) specific for Exon 1. Nucleotide and amino acid sequences were analyzed and aligned using either the Clustal method and Lasergene software (DNAStar Inc., Madison, WI) or the PILEUP and ALSCRIPT (Genetics Computer Group, Madison, WI) sequence analysis package (Version 9.0). Transcription factor binding site homologies were identified in promoter DNA sequences by searching the transcription factor database using the GCG program.

RNA gel blot analysis

For RNA analysis, roots and other tissues were harvested from mature wild-type (HP; *Nic1Nic2*) and low alkaloid mutant (LP; *nic1nic2*) Burley 21 tobacco plants. For topping experiments, the stem was cut and the top one-third of the plant was removed just prior to flower opening. Roots were harvested just prior to topping (0 hr control) and at various times after decapitation. The tissue was immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction and isolation.

Total RNA was isolated from vegetative organs and floral structures of HP and LP Burley 21 tobacco using the TRI-reagent (Molecular Research Center Inc., Cincinnati, OH) and quantified spectrophotometrically by measuring *A*260. Total RNA (5 µg) was electrophoresed through 1.2% agarose gels (containing 0.4 M formaldehyde) and transferred to Nytran+ nylon membranes. Following prehybridization the membranes were hybridized with a single-stranded *NtPMT1a* cDNA antisense probe (corresponding to the antisense strand of Exons 2 to 8 of the *NtPMT1a* cDNA coding region) as described above. As a control to quantify and normalize RNA levels in each lane, the blot was hybridized with a 400-bp probe derived from the β-ATPase cDNA using primers 6 and 7 (Table 1) as described below.

Semi-quantitative RT-PCR analysis of individual PMT transcript levels

Total RNA (1 µg) extracted from the roots of HP and LP Burley 21 tobacco plants was reverse-transcribed into first-strand cDNA at 42°C using Superscript II reverse transcriptase (Gibco BRL) according to the manufacturer's protocol. Two gene-specific primers were employed in the reactions: primer 5 capable of recognizing Exon 3 of the *PMT* genes and primer 8 specific for Exon 8 of the nuclear gene encoding the β-subunit of mitochondrial ATPase from *N. plumbaginifolia* (*NpATP2.1*) and *N. sylvestris* (*NsATP2.1*) (Boutry and Chua, 1985; Lalanne *et al.*, 1998). The β-ATPase transcript served as an internal reference (constitutively-expressed control) to determine loading accuracy and to normalize expression levels (Kinoshita *et al.*, 1992) Following first strand cDNA synthesis, two sets of nested primers (0.4 µM each primer) were used to amplify the *PMT* and *β-ATPase* transcripts: primers 1 and 2 (Table 1) recognized Exon 1 in all five *PMT* transcripts and gave products ranging in size from 220 bp to 420 bp and primers 6 and 7 amplified an approximately 400-bp region encompassing a portion of Exons 6 to 8 of the β-ATPase coding region. Amplification was carried out for 25 cycles using the following reaction conditions: denaturation at 95°C for 1 min, primer annealing at 60°C for 35 sec, and extension at 72°C for 1.5 min; a final extension was conducted at 72°C for 6 min. Amplification products were radioactively labeled by spiking the PCR reaction with 10 µCi 32P-dCTP. Aliquots of the PCR reaction were analyzed on a 6.5% non-denaturing polyacrylamide/1X TBE gel and electrophoresed at 600 volts. The reaction conditions were optimized to provide amplification of both PMT and $-ATPase transcripts in the linear range of the reaction by varying the levels of first strand cDNA template, annealing temperature, and number of cycles of amplification as described in Kinoshita *et al.* (1992). Molecular weight standards were prepared by PCR amplification using the same primers and protocol described above and plasmid DNA templates containing the PMT encoding genomic fragments, as well as genomic DNA from the various *Nicotiana* species indicated in the text.

Following electrophoresis, the polyacrylamide gels were fixed in 5% MeOH, 7.5% acetic acid for 30 min, dried overnight, and used to expose X-ray film. PMT band intensities were quantified using phosphorimager analysis (Molecular Dynamics) and normalized relative to the intensities of the β-ATPase control band in each lane. The experiment was conducted twice with different total RNA samples, and representative results are presented from one of the two experiments.

Results

PMT gene structure and organization in N. tabacum

Gel blot analysis of total genomic DNA isolated from *N. tabacum* cv. Xanthi, hybridized with a radioactively-labeled cDNA (*NtPMT1a*) encoding the complete coding region of putrescine N-methyltransferase (PMT) showed the presence of five major hybridizing bands in *Kpn*I or *Eco*RI digested DNA, consistent with the presence of a small multigene family in the *N. tabacum* genome (Figure 1).

As part of our initial characterization of the gene family encoding PMT in *N. tabacum*, an EMBL3 genomic library, prepared from *N. tabacum* cv. Xanthi DNA, was screened using the *NtPMT1a* (A411 homologous) cDNA as probe. From a total of 18 recombinant phage isolated, three phage were recovered that contained genomic fragments encoding the *NtPMT2*, *NtPMT3* and *NtPMT4* genes. The three *PMT* genes were completely encoded within a unique sized *Eco*RI fragment within the phage DNA insert which allowed for the correlation of each with a hybridizing restriction fragment on the gel blot of *N. tabacum* genomic DNA (Figure 1). The complete coding region and immediate 5' and 3' non-coding sequences of the three genes were determined and found to encode full-length PMT proteins (Figure 2). Each *PMT* gene consisted of 8 exons and 7 introns, consistent with the gene structure reported previously for the *PMT* genes from *N. sylvestris* (Hashimoto *et al.*, 1998a). Comparison of the deduced amino acid sequences (Figure 2) revealed that the encoded PMT proteins were extremely similar over their entire length, with the only significant variability in primary sequence localized to the extreme N-terminal region of the protein. This region, completely encoded within Exon 1, contains a variable number of an 11 amino acid repeat with a consensus sequence of NGHQNGTSEHQ. The function of the repeated sequence is unknown, but is apparently inconsequential to enzyme function, since the number of repeats does not influence activity and PMTs characterized from other species do not contain the repeated element (Hashimoto *et al.*, 1998a; Suzuki *et al.*, 1999a).

Multiple rounds of screening of the EMBL3 genomic library failed to yield additional hybridizing phage containing sequences encoding the other two *PMT* genes thought to be present in the *N. tabacum* genome and, therefore, a directed cloning approach was pursued using a subgenomic library constructed from *Eco*RI fragments isolated from *N. tabacum* cv. Xanthi. From this hybridization screening, a phage containing the approximately 3.1 kb EcoRI fragment encoding *NtPMT1a* was recovered. The coding region of the *NtPMT1a* gene was found to be identical to the A411 cDNA (Hibi *et al.*, 1994), with the exception of a single base change in Exon 6 that results in a conservative amino acid substitution. This difference could be the result of minor differences among cultivars used in the two studies (i.e., Xanthi vs. Burley 21). Translation of the open reading frame contained in *NtPMT1a* showed that it encoded a protein containing four N-terminal 11 amino acid repeats, similar to Exon 1 of the *PMT* gene present in *N. tomentosiformis* (Hashimoto *et al.*, 1998a).

Given the observation that *NtPMT1a* encoded a homolog of the *PMT* gene present in *N. tomentosiformis*, the nature and possible evolutionary origin of the remaining *PMT* gene present in the *N. tabacum* genome was brought into question. From our expression studies (described in detail below), we had determined that five distinct PMT encoding transcripts were present in the roots of *N. tabacum*, four of which could be accounted for based upon the length of the Exon I coding region in the four *PMT* genes isolated and characterized in our studies described above. The fifth transcript was similar in size to that encoded by *NtPMT1a* and, therefore, was designated *NtPMT1b*. Since the variability in *PMT* gene structure is primarily localized within Exon 1, we used a PCR-based strategy to analyze the *PMT* gene structure and family size in *N. otophora*, the other possible progenitor of *N. tabacum*. As shown in Figure 3, five distinct PCR products were detected in the electrophoretic pattern of amplification products generated from *N. tabacum* genomic DNA using Exon 1 specific primers (Table 1). Consistent with our studies described above and the previous work of Hashimoto *et al.* (1998a), three PCR products were detected in the electrophoretic pattern of amplification products generated from *N. sylvestris* genomic DNA, and a single band was recovered from *N. tomentosiformis* genomic DNA. Amplification of genomic DNA from *N. otophora* using Exon 1 specific primers also yielded only a single band, whose electrophoretic mobility was most similar to that of the *NtPMT1b* derived product.

Analysis of PMT gene intron and flanking sequences

The location of the seven introns within the protein coding region of the five *PMT* genes in *N. tabacum* is identical and appears to be conserved among *PMT* genes from different *Nicotiana* species. There is also little variation in the nucleotide sequences that comprise the Exon-Intron splice junctions in the various *PMT* genes in *N. tabacum* (Table 2). The high degree of nucleotide sequence similarity recognized among *PMT* genes within their coding regions is also present within their introns and immediate 5' and 3' flanking sequences (Table 2 and Figure 4). In general, a greater level of sequence identity is found in the introns of the *NtPMT2*, *NtPMT3*, and *NtPMT4* genes, than in pair-wise comparisons among the introns of the other members of the *N. tabacum PMT* gene family. The observed conservation in the intron sequences of the *NtPMT2*, *NtPMT3*, and *NtPMT4* genes is consistent with their origin from the same progenitor species (*N. sylvestris*). One interesting exception occurs within Intron 6, where the length of the intron and the sequence similarity is more conserved between *NtPMT1a* and *NtPMT4*, than between *NtPMT4* and *NtPMT2* or *NtPMT3*.

Approximately 1 kb of nucleotide sequence was determined 5' to the coding regions of the *NtPMT1a*, *NtPMT2*, *NtPMT3*, and *NtPMT4* genes (Figure 4). By comparison to the 5'-untranslated

region (UTR) present in the A411 cDNA, we set the start site for transcription initiation at approximately 57 nucleotides upstream of the MET start codon in *NtPMT1a* and *NtPMT3,* and either 69 or 60 nucleotides upstream in *NtPMT2* and *NtPMT4*. The major distinguishing feature between the 5'-UTRs in the various genes is the presence or absence of a 17 bp sequence in the gene. An appropriately placed TATA-box can be easily recognized 45 bp 5' to the initiation site in all four genes. Within the first 200-250 bp upstream of the TATA box, a high level of sequence conservation is found to exist among the promoter regions in the four genes. After this point, a clear difference can be observed between the *NtPMT1a* promoter and the remaining three genes, and by 400 bp upstream, little similarity can be found among any of the gene family members.

Analyzing the proximal regions of the various *PMT* promoters with various motif scanning software identified several G-box-like sequences (Foster *et al.*, 1994; Kim *et al.*, 1992; Menkens *et al.*, 1995; Staiger *et al.*, 1989; Williams *et al.*, 1992) at various positions among the *PMT* promoters, and a potential metal response element (MRE) (positions –75 to –66; numbering relative to the *NtPMT1a* promoter sequence) in three of the four *PMT*s (Cizewski-Culotta and Hamer, 1989; Thiele, 1992). An unusual 17 nucleotide stretch of guanine occurs at positions -259 to -243 in the *NtPMT1a* gene promoter followed upstream by a purine-rich region (positions –332 to –263). In the *NtPMT3* promoter a 14 bp palindromic sequence (positions -497 to -484) was detected. *PMT* gene expression has been reported to increase in root tissues following treatment with methyl jasmonate (Imanishi *et al.*, 1998). However, none of the sequence motifs reported to confer methyl jasmonate-responsiveness in other plant genes (Mason *et al.*, 1993; Rouster *et al.*, 1997) were detected in the *PMT* promoters.

Comparison of the available nucleotide sequence information from the 3'-flanking regions of the various *PMT* genes in *N. tabacum* revealed that the 3'-UTRs in the *NtPMT2*, *NtPMT3*, and *NtPMT4* genes of *N. tabacum* share approximately 81-94% identity with each other and are essentially identical to those reported for *N. sylvestris* PMTs by Hashimoto *et al.* (1998a). The major distinguishing feature among the various genes is the presence of two short (20 bp and 4 bp) deletions in the *NtPMT2* gene, which lowers the percent identity. The 3'-UTR of *NtPMT1a* is identical to that reported for the A411 cDNA (Hibi *et al.*, 1994) and 81-94% identical to the other *PMT* genes in the *N. tabacum* genome. Unfortunately, no sequence information is currently available for the 3'-UTR of the *N. otophora* or *N. tomentosiformis PMT* genes.

Regulation of PMT gene expression

To determine whether the members of the *PMT* gene family in *N. tabacum* were differentially

expressed, a series of experiments were carried out to define the temporal and spatial distribution of transcripts arising from the five genes. Shown in Figure 5A are the results of gel blot analysis of total RNA extracted from various tissues of mature Burley 21 tobacco plants hybridized with radioactively-labeled probe capable of detecting all five *PMT* transcripts. Consistent with previous studies (Hashimoto *et al.*, 1998b; Hibi *et al.*, 1994), *PMT* expression is localized exclusively to roots. When maturing wild-type (HP) Burley 21 plants are topped (i.e., the floral meristem and upper 1/3 of the stem are removed), a dramatic increase in *PMT* transcript abundance is observed within 2 hr, reaching a maximal level of accumulation by 12-24 hr. Two size transcripts are detected on the gel blots, reflecting the small difference in message size that occurs as a result of the difference in size of Exon 1 among the genes.

In addition to examining *PMT* gene expression in wild-type plants, we also examined expression in a low nicotine-producing (LP) mutant of Burley 21 (Legg and Collins, 1971). The low nicotine Burley 21 line harbors mutations at two independent loci (*nic1* and *nic2*) thought to be global regulators of gene expression involved in alkaloid formation. As shown in Figure 6B, topping of the low nicotine mutant (*nic1nic2*) Burley 21 did not cause an increase in *PMT* transcript abundance as observed in wild type plants. Thus, it appears that *Nic1* and *Nic2* are likely involved in regulation of *PMT* expression in the very least, and may also be involved in the regulation of other genes in the alkaloid biosynthetic pathway. Whether this is a direct effect (e.g., transcriptional activation) or indirect remains to be determined.

In order to determine the extent to which the individual members of the gene family contributed to the general pattern of expression described above, a semi-quantitative RT-PCR strategy (Kinoshita *et al.*, 1992) was used to detect and quantify the levels of the individual *PMT* transcripts in the roots of both wild-type (HP) and low alkaloid (LP) Burley 21 tobacco. This approach takes advantage of the fact that Exon 1 is variable in length among the various *PMT* genes (Figure 2), allowing for their individual detection and quantitation following polyacrylamide gel electrophoresis and autoradiography.

Five RT-PCR products (representing Exon 1 from each of the five genes present in *N. tabacum*) were detected in the electrophoretic profiles of amplification products derived from reactions using either HP or LP Burley 21 root RNA (Figure 6A). All five *PMT* genes present in the *N. tabacum* genome were expressed in the roots of wild-type plants, and topping resulted in a differential accumulation of transcripts derived from each gene. Among the five genes, transcripts derived from the *NtPMT2* and *NtPMT1b* showed the greatest increase in abundance rising approximately 3-fold during the first 24 hr post-topping, whereas levels of the *NtPMT1a* and *NtPMT4* transcripts changed little in response to topping (Figure 6B). In the LP mutant, little or no effect was observed on the

levels of the various *PMT* transcripts following topping, although in some cases (e.g., *NtPMT1a*) a small but likely insignificant depression in transcript abundance was detected. Thus, it appears that all five genes contribute to PMT activity levels within the root.

II. ADC

The present invention features the characterization of two members of the nuclear gene family encoding ADC in tobacco *N. tabacum* L. As the following discussion shows, *ADC2* is preferentially expressed in roots and accounts for the major portion of *ADC* transcripts present. Furthermore, analysis of *ADC* transcript levels in roots of low and high nicotine producing lines showed that *ADC* expression is under the control of the *Nic1 Nic2* regulatory loci.

Materials and methods

Plant growth and tissue preparation

Seeds of *N. tabacum* cv. Xanthi, wild-type and low alkaloid *nic1 nic2* mutant *N. tabacum* cv. Burley 21 were obtained from Dr. G. Collins (University of Kentucky, Lexington). Tobacco plants used for DNA isolation were grown in soil:vermiculite mixture in the greenhouse under natural lighting conditions. Plants used for RNA extraction were grown either in Moltan Plus (Moltan Co., Middleton, TN) or hydoponically in a dilute (half-strength) Peters nutrient solution with continuous aeration of the roots under natural lighting conditions in the greenhouse. Topping experiments were conducted by removing the floral meristem, leaves and stem (approximately the upper 1/3 of the plant) from tobacco plants just prior to blooming. Plant tissues were collected from fully matured individuals, frozen in liquid nitrogen, and stored at -80°C until used for RNA preparation (see below).

Screening of genomic libraries and phage characterization

A genomic library constructed in λ EMBL3 from *N. tabacum* cv. Xanthi leaf DNA (Clonetech, Inc., Palo Alto, CA) was screened by plaque hybridization (Sambrook *et al.*, 1989) using an [α- ^{32}P]-dCTP-labeled, 2.7 kb *Eco*RI-*Xho*I fragment from plasmid PR24 as probe. PR24 encodes a full length ADC cDNA isolated from the roots of wild-type *N. tabacum* cv. Burley 21 (Wang, 1999). Hybridization was performed at 65°C for 16 h in a solution containing 0.25 M Na_2HPO_4 (pH 7.2) and 7% (w/v) SDS. Following hybridization, the membranes were washed twice in 2 x SSC, 0.1%

SDS for 15 min at room temperature, once in 0.2 x SSC, 0.1% SDS for 30 min at 65°C. Hybridizing phage were picked and plaque purified through three subsequent rounds of hybridization screening. Phage DNA was isolated from plaque purified phage using a Qiagen Phage Midi Preparation Kit (Qiagen, Germany) and insert DNA characterized by restriction mapping and DNA gel blot analysis. The relevant hybridizing bands in each phage were cloned into pBluescript SK+ vectors for further analysis.

Nucleic acid sequencing and analysis

Nucleotide sequencing was carried out manually using the Sequenase Version 2.0 protocols according to the manufacturer's protocol (United States Biochemical, Cleveland, OH) or with an ABI 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA) using double-stranded plasmid DNA templates prepared utilizing the Qiaprep Spin Plasmid Kit (Qiagen USA, Valencia, CA). The nucleotide and predicted amino acid sequences of the various cDNAs were analyzed using BLAST sequence analysis programs (Altschul *et al.*, 1990; Gish and States, 1993) and protein sequence alignments were carried out using the PILEUP program (Genetics Computer Group Sequence Analysis package, Version 9.0 (GCG, University of Wisconsin, Madison, WI) and the various gene sequences available in the NCBI (National Center for Biotechnology Information, Bethesda, MD) nucleotide and protein sequence database. Manual adjustment of the sequence alignments were carried out as necessary.

RNA isolation and gel blot analysis

Total RNA was extracted from tobacco roots, leaves, and floral parts using Tri-Reagent (Molecular Research Center, USA, Cincinnati, OH) according to the manufacturer's protocol. For RNA gel blot analysis, aliquots (10 µg) of total RNA extracted from the various tissues were fractionated by electrophoresis through a 1.2% agarose-formaldehyde gel and blotted onto Nytran nylon membranes (Schleicher & Schuell, Keene, NH) using 10 X SSC. The transferred RNA was UV cross-linked to the membrane using a UV Stratalinker (Stratagene, La Jolla, CA) and the membranes were prehybridized in 7% SDS, 0.25 M Na_2HPO_4, pH 7.2 for 2-4 hours at 65°C. Hybridization was carried out in the same buffer in the presence of ^{32}P-labeled probes for 16 hr at 65°C. The membranes were washed under high stringency conditions and subject to autoradiography at -80°C for approximately 48 h.

For gel blot analysis, [α-^{32}P]-dCTP -labeled probes were prepared by random primed labeling

(Random Primed Labeling Kit, Boehringer Mannheim, Indianapolis, IN) using 25-50 ng of a 2.7 kb *Eco*RI-*Xho*I fragment derived from PR24 and a 460 bp fragment amplified from the β- subunit of the tobacco mitochondrial ATP synthase gene (*atp2*) (Boutry and Chua, 1985).

Semi-quantitative RT-PCR analysis of NtADC1 and NtADC2 transcript levels.

Total RNA (2 μg) from roots, leaves, or floral parts was reverse transcribe at 40°C for 1 h in a reaction cocktail containing 200 units of SuperscriptII reverse transcriptase (RNase H-, Gibco BRL, USA), 10 units RNase inhibitor (Perkin Elmer), 200 μm dNTPs and 40 pmol of primer, in total volume of 20μl. For first strand cDNA synthesis, a single primer [5'-AGAAAAACATCACCAACT-3'] capable of hybridizing to both the *ADC1 and ADC2* transcripts was used in the reaction. As a control, a primer (5'-GCAACTGTCATCTTATCATCTTC-3') specific for the β-subunit of the tobacco mitochondrial ATP synthase gene *apt2* (Boutry and Chua, 1985) was used in the reverse transcriptase reaction.

Following reverse transcription, the single stranded cDNA products were serially diluted over a concentration range between 1 to 50 ng RNA, and PCR amplification was carried out for 25 cycles of 45 s at 94°C, 1 min at 64°C and 1 min at 72°C in a Genemate thermocycler (ISC Bioexpress, UT). The reaction mixture contained cDNA template, 1 x PCR buffer (Boehringer Mannheim), 100 μM dNTPs, 25 pmol of each forward and reverse primer and 1 unit Taq DNA polymerase. The PCR reactions specific for *ADC1* transcripts contained the following primers: ADC1-forward, 5'-CGTAGACGCTACTGTTTC-3' and ADC1-reverse, 5'-TGGACAAC TGTGGAGGCG-3'. Reactions specific for *ADC2* transcripts contained primers ADC2-forward, 5'-TGTAGATGCTGCTGTTGTTT-3', and ADC2-reverse, 5'-TGAACAAC TGCGGAGGCA-3'. Control reactions for normalization of amplification products contained 25 pmol of primers specific for the tobacco *apt2* transcripts: *atp2* forward, 5'-GTATATGGTCAAATGAATGAGCC-3', and *atp2* reverse.int, 5'-GCAGTATTGTAGTGATCCTCTCC-3'. For quantitation purposes, amplification reactions were supplemented with 1μCi ^{32}P-dCTP. PCR products were separated by electrophoresis through 1.2% agarose gels, the fractionated reaction products transferred onto a Hybond N+ membranes, dried and subject to autoradiography at -70° C. Quantitation was carried out by phosphorimaging using a Molecular Dynamics PhosphorImager. Values were normalized relative to the intensities of the *atp2* control band in each lane. The experiment was conducted twice with different total RNA samples, and representative results are presented from one of the two experiments.

Results

These studies show the structure and expression of individual members of the *ADC* gene family in tobacco. An α- ^{32}P-dCTP-labeled 2.7 kb EcoRI-XhoI fragment from PR24 encoding the ADC coding region was used to screen an λ EMBL3 phage genomic library. From a screen of approximately 3 X10^5 phage, seventeen hybridizing phage were recovered, of which five were fully characterized by restriction mapping and DNA gel blot analysis. These phage fell into two groups based on their restriction profile. The relevant hybridizing fragments from the various phage were cloned into pBluescript and their nucleotide sequence determined.

Presented in Figure 7 are the nucleotide and predicted amino acid sequences of NtADC-1 and NtADC-2 genes. Both genes contain a single open reading frame, uninterrupted by introns. The nucleotide and amino acid sequence encoded in NtADC-1 is identical to that of PR24, the full length cDNA isolated from *N. tabacum* cv Burley 21. There are 84 nucleotide differences within the NtADC-1 and NtADC-2 coding regions, resulting in 23 amino acid differences between the ADC1 and ADC2 proteins, respectively. The ADC1 protein is one amino acid shorter in length, missing Val-13.

By comparison to the full-length cDNA, the 5'-untranslated region (UTR) present in NtADC-1 and NtADC-2 are 431 bp and 432 bp long, respectively. The size of the 5'-UTR in the ADC transcripts is considerably larger than the average size of the plant leader sequence (Joshi, 1987). In contrast, the 3'-UTRs present in NtADC-1 and NtADC-2 are relatively short, approximately 84 nucleotides in length. In both gene sequences, a conserved polyadenylation signal (AATAATA) can be recognized 23 nucleotides from the site of polyadenylation site found in the PR24 cDNA.

Pairwise comparison of the *N. tabacum* ADC1 and ADC2 proteins with the ADCs of other plant species showed that the *N. tabacum* proteins are approximately 82% identical to the ADC of its evolutionary progenitor species *N. sylvestris* [Genbank Accession No. AB012873] and 86% identical to the ADC from tomato (*Lycopersicon esculentum*) [31], another member of the Solanaceae family (Figure 2). As might be expected, the *N. tabacum* ADC shares considerably less similarity to ADCs isolated from species more distantly related evolutionarily, such as *Arabidopsis* - 67% identical [32, 33], soybean- 67% identical [34], and oat - 42% identical [35] and is only 29% identical to the enzyme from *Escherichia coli* - [36].

The predicted protein coding regions for the *N. tabacum* ADCs are substantially longer than those reported for the ADC proteins of *N. sylvestris* and *L. esculentum* [31], but are similar in length to those reported in *Arabidopsis*, oat, soybean [32-35] and for the *E. coli* enzyme [36]. The

difference in overall length appears to arise from an apparent nucleotide deletion in the *N. sylvestris* and tomato cDNA sequences relative to the ADC1 and ADC2 predicted sequence and those in other plants. In the nucleotide sequences reported for both the *N. sylvestris* and tomato cDNAs, a guanine residue (position 2295 in the *N. sylvestris* sequence and 1531 in the tomato sequence) is missing [Genbank Accession No. AB012873]. This deletion changes the reading frame and introduces a premature termination to the predicted coding region. Using the sequence information available in the NCBI database, correcting for this error allowed us to extend the predicted C-terminus of the both ADC proteins, yielding the alignment to the *N. tabacum* ADCs and those of other plant ADCs as indicated in Figure 8. We have also included in the alignment shown in Figure 8, the correction at the N-terminus of the predicted tomato ADC protein sequence noted by Pérez-Amado et al. [37], allowing better alignment of all of the higher plant sequences.

Developmental regulation of arginine decarboxylase expression

It has been shown that nicotine formation can be activated in the roots of maturing tobacco plants by topping, that is, removal of the flower head and several young leaves (Akehurst, 1981; Hibi, et al., 1994). Coincident with the activation of nicotine formation, there is an increase in the levels of transcripts encoding ODC, PMT and spermidine synthase (SPS) over the subsequent 24 hr period in wild-type plants (Hibi et al., 1994; Riechers and Timko, 1999). To determine the effects of topping on *ADC* expression in roots, Burley 21 plants were grown in the greenhouse to the bud stage at which point the upper 1/3 of the plant was removed and samples of roots tissues were collected before and at various times post-topping. As shown in Figure 9, *ADC* message abundance increased in the roots of topped Burley 21 plants during the 24 hr period after topping. Low alkaloid (LA) mutants of Burley 21 show a much lower level of ADC expression in their roots, and no induction of ADC transcript accumulation after topping. The lack of ADC induction in the low-alkaloid mutant is consistent with previous studies (Hibi *et al.*, 1994; Riechers and Timko, 1999; Wang, 1999) showing a general inability to activate gene expression leading to increased polyamine formation and alkaloid biosynthesis as a result of the mutation of the *Nic1* and *Nic2* regulatory genes.

NtADC-2 is predominately expressed in roots of wild-type plants.

Due to the high degree of identity between the NtADC-1 and NtADC-2 transcripts (e.g., 95.8% coding regions, 94.4% and 96.4% in 5'- and 3'-UTRs, respectively), it is impossible to distinguish between the two transcripts by RNA gel bot analysis. Therefore, we employed a RT-PCR based

strategy and gene specific oligonucleotide primers. Total RNA was extracted from tobacco roots, leaves and flowers, and single-stranded cDNA synthesized using an oligonucleotide primer capable of hybridizing to both ADC1 and ADC2 transcripts. As an internal control for amplification, a gene specific primer recognizing the *atp2* transcript encoding the β-subunit of the tobacco mitochondrial ATPase was include in the reactions. Under experimental conditions providing amplification in the linear range of the PCR reaction, gene specific forward and reverse primers were used to specifically amplify either ADC1 or ADC2 cDNAs. Test reactions (Figure 10A) using plasmid DNA encoding NtADC1 or NtADC2 as template demonstrated the specificity of the primers. As shown in Figure 10B, the main transcripts detectable in all tissues tested are derived from NtADC-2. Flowers express the highest level of ADC, and leaves lowest. In the flowers, although ADC1 is detectable, far less than ADC2 Roots also express a significant level of ADC.

ADC transcript levels are highest in the roots and floral organs, and low in other plant tissues. The two ADC genes investigated appear to have different modes of regulation, with ADC2 being predominately expressed in the roots and other organs.

At the present time, only limited information is available on the nature of regulatory regions in the promoters of genes encoding enzymes of alkaloid biosynthesis. The availability of cloned genomic fragments encoding ADC allows one to begin mapping regulatory sequences within members of these genes responsible for tissue specific, developmental, and inducible expression.

III. ODC

The present invention features the genes of two members of the nuclear gene family encoding ODC in tobacco *N. tabacum*. As the following experimental discussion shows, the ODC-2 gene is preferentially expressed in roots and floral tissues. Furthermore, the abundance of ODC transcripts in root tissues is affected by topping. Furthermore, analysis of ODC transcript levels in roots of low and high nicotine producing lines shows that ODC expression is under the control of the *Nic1 Nic2* regulatory loci.

Materials and methods

Plant growth and tissue preparation

Seeds of *N. tabacum* cv. Xanthi, wild-type and low alkaloid *nic1 nic2* mutant *N. tabacum* cv. Burley 21 were obtained from Dr. G. Collins (University of Kentucky, Lexington). Tobacco plants used for DNA isolation were grown in soil:vermiculite mixture in the greenhouse under natural lighting

conditions. Plants used for RNA extraction were grown either in Moltan Plus (Moltan Co., Middleton, TN) or hydroponically in a dilute (half-strength) Peters nutrient solution with continuous aeration of the roots under natural lighting conditions in the greenhouse. Topping experiments were conducted by removing the floral meristem, leaves and stem (approximately the upper 1/3 of the plant) from tobacco plants just prior to blooming. Floral parts and other plant tissues were collected from fully matured individuals, frozen in liquid nitrogen, and stored at -80°C until used for RNA preparation (see below).

Screening of genomic libraries and phage characterization

A genomic library constructed in EMBL3 from *N. tabacum* cv. Xanthi leaf DNA (Clonetech, Inc., Palo Alto, CA) was screened by plaque hybridization (Sambrook *et al.*, 1989) using a ^{32}P-radiolabeled, 1.6 kb *Eco*RI-*Xho*I insert from plasmid PR46 as probe. PR46 encodes a full length ODC cDNA previously isolated by differential screening of plasmid libraries prepared from mRNA isolated from the roots of wild-type Burley 21 plants before and 3-days post-topping (Wang, J., Sheehan, M., Bookman, H. and Timko, M.P., unpublished data). Hybridization was performed at 65°C for 16 h in a solution containing 0.25 M Na_2HPO_4 (pH 7.2) and 7% (w/v) SDS. Following hybridization, the membranes were washed twice in 2 x SSC, 0.1% SDS for 15 min at room temperature, once in 0.2 x SSC, 0.1% SDS for 30 min at 65°C. Hybridizing phage were picked and plaque purified through three subsequent rounds of hybridization screening. Phage DNA was isolated from plaque purified phage using a Qiagen Phage Midi Preparation Kit (Qiagen USA, Valencia, CA) and insert DNA characterized by restriction mapping and DNA gel blot analysis. The relevant hybridizing bands in each phage were cloned into pBluescript SK+ vectors for further analysis.

Nucleic acid sequencing and analysis

Nucleotide sequencing was carried out manually using the Sequenase Version 2.0 protocols according to the manufacturer's protocol (United States Biochemical, Cleveland, OH) or with an ABI 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA) using double-stranded plasmid DNA templates prepared utilizing the Qiaprep Spin Plasmid Kit (Qiagen USA, Valencia, CA). The nucleotide and predicted amino acid sequences of the various cDNAs were analyzed using BLAST sequence analysis programs (Altschul *et al.*, 1990; Gish and States, 1993) and protein sequence alignments were carried out using the PILEUP program (Genetics Computer Group Sequence Analysis package, Version 9.0 (GCG, University of Wisconsin, Madison, WI) and the various gene sequences available in the NCBI (National Center for Biotechnology Information, Bethesda, MD) nucleotide and protein sequence database. Manual adjustment of the sequence alignments were

carried out as necessary.

RNA isolation and gel blot analysis

Total RNA was extracted from tobacco roots, leaves, and floral parts using Tri-Reagent (Molecular Research Center, USA, Cincinnati, OH) according to the manufacturer's protocol. For RNA gel blot analysis, aliquots (10 µg) of total RNA extracted from the various tissues were fractionated by electrophoresis through a 1.2% agarose-formaldehyde gel and blotted onto Nytran nylon membranes (Schleicher & Schuell, Keene, NH) using 10 X SSC. The transferred RNA was UV cross-linked to the membrane using a UV Stratalinker (Stratagene, La Jolla, CA) and the membranes were prehybridized in 7% SDS, 0.25 M Na_2HPO_4, pH 7.2 for 2-4 hours at 65°C. Hybridization was carried out in the same buffer in the presence of ^{32}P-labeled probes for 16 hr at 65°C. The membranes were washed under high stringency conditions and subject to autoradiography at - 80°C for approximately 48 h.

Restriction fragments derived from cDNA clones of interest were separated by agarose gel electrophoresis, the DNA was purified, and quantified by spectrophotometry. [^{32}P]-dCTP -labeled probes were prepared from 25-50 ng of insert DNA by random primed labeling (Random Primed Labeling Kit, Boehringer Mannheim, Indianapolis, IN). As a control, the blots were also probed with radioactively labeled probes encoding the alkaloid biosynthesis enzyme putrescine N-methyltransferase (PMT) (Riechers and Timko, 1999), a root specific, topping inducible β-glucosidase encoding cDNA (TBG-1) (Riechers, D.E. and Timko, M.P., unpublished data), 26S rRNA (PR31) or 28S rRNA fragments.

Genomic DNA isolation and gel blot analysis

Tobacco genomic DNA was prepared from tobacco leaf tissue by the method of Junghans and Metzlaff (1990). Total genomic DNA (15 µg) was digested to completion with *Eco*RI or *Hind*III, the digestion products were fractionated by electrophoresis through a 0.8% (w/v) agarose gel, and transferred onto Nytran nylon membrane (Schleicher & Schuell, Keene, NH) in the presence of 0.4 N NaOH (Sambrook *et al.*, 1989). Following transfer, the membrane was rinsed in 2 X SSC , the DNA was UV cross-linked to the membrane, and the membrane was prehybridized and hybridized as described above. Following hybridization and washing, the membranes were subjected to autoradiography at –80°C.

Results and discussion

Gel blot analysis of tobacco genomic DNA cut with various restriction enzymes and hybridized with an [α- ^{32}P]-dCTP-labeled 1.6 kb *Eco*RI-*Xho*I cDNA fragment (PR46) encoding the full-length ODC protein from *N. tabacum* cv Burley 21 (Wang, J., Sheehan, M., Bookman, H. and Timko, M.P., unpublished data) indicated ODC is encoded by small gene family in the *N. tabacum* genome (Fig. 11). Four to five major bands and several minor bands of sufficient size to encode full-length genes are detected in either *Eco*RI or *Hind*III digested tobacco DNA.

To further analyze the structure and regulation of members of the *ODC* gene family in tobacco, a λ EMBL3 phage genomic library constructed with DNA from *N. tabacum* cv Xanthi was screened using a [α- ^{32}P]-labeled probes prepared from PR46 (as described above). From a screen of approximately 3 X10^5 phage, five hybridizing phage were recovered, of which three were fully characterized by restriction mapping and DNA gel blot analysis. Two phage proved to contain identical insert DNA and the third had a unique restriction digestion profile. Following DNA gel blot analysis, the hybridizing fragments were cloned into pBluescript and their nucleotide sequence determined.

The complete *NtODC-2* gene spans two *Sal*I fragments of 2.7 kb and 6.5 kb. The coding region of the gene contains a single1302 bp open reading frame uninterupted by introns (Fig. 12). The nucleotide sequences of *NtoDC-2* is identical within the coding and 5' and 3'- untranslated regions to the PR46 encoded cDNA, with the exception of four nucleotide changes (residues +2, +4, +6 and +8) in the 5'-untranslated region. These nucleotide differences likely reflect changes introduced during the cDNA synthesis reaction.

The predicted amino acid sequence for the *NtODC-2* encoded protein (designated pODC2) (Fig. 13) is identical to the ODC characterized from Burley 21 tobacco encoded by PR46 (Wang, J., Sheehan, M., Bookman, H. and Timko, M.P., unpublished data) and to the partial *N. tabacum* ODC cDNA sequence (PR17) reported by Malik *et al.*, (1996). Comparison of the predicted amino acid sequence for pODC2 with the ODC proteins characterized from two different tobacco cultivars showed that the pODC2 differs by 7 amino acid (98% identity) from the ODC protein characterized from the high alkaloid cultivar, *N. tabacum* cv. SC58 [Genbank Accession No. Y10472.1] and by 7 amino acid (98% identity) from ODC protein from BY-2 cells. The tobacco pODC2 is 89% and 90% identical to the ODCs from tomato (*Lycopersicon esculentum*) and jimsonweed (*Datura stramonium*), respectively, but substantially less similar to ODCs from yeast (35% identity) and humans (32% identity).

The *NtODC-1* gene, contained on an 4.0 kb *Xba*I fragment, encodes a single open reading frame of 141 amino acids encompassing the amino terminal one-half of ODC (Fig. 12). Six amino acid residue changes distinguish the *NtODC-2* and *NtODC-1* encoded proteins over the homologous

region of the proteins. Beginning at amino acid residue 130, the *NtODC-1* encoded protein (pODC1) diverges from pODC2, with a stop codon present after residue 141. Scanning the available nucleotide sequence (> 1 kb) in the 3'-flanking region of the NtODC-1 gene failed to reveal any evidence for ODC homologous protein sequences in any of the three translational reading frames. Interestingly, a comparison of the 5'-flanking sequence of the *NtODC-1* and *NtODC-2* genes revealed that while the *NtODC-2* gene has a clearly recognizable TATA-box properly located at approximately -35 bp from the transcriptional start site, no such regulatory motif is found in the *NtODC-1* gene sequence. Consistent with this observation, RNA gel blot analysis performed using a hybridization probe prepared from *NtOCD-1* immediately downstream of the frame shift, failed to detect any message in various tissues of mature tobacco plants (data not shown). Thus, it appears that *NtODC-2* represents an unexpressed pseudogene in the *N. tabacum* genome.

To determine the spatial pattern of expression of the *NtODC-2* gene, gel blot analysis was carried out using total RNA prepared from roots, stems, young and mature leaves, and various floral parts of Burley 21 tobacco plants. As shown in Fig 14, transcripts encoding ODC were easily detected in the roots, with little or no expression in other tissues except sepals, carpels, and mature stamens.

The formation of nicotine and total leaf alkaloids in tobacco is known to be under the control of at least two independent genetic loci (Legg *et al.*, 1969; Legg and Collins, 1971), designated *Nic1* and *Nic2* (Hibi *et al.*, 1994). *Nic1* and *Nic2* are semidominant and operate synergistically to control plant alkaloid content, with mutations within these genes resulting in plants with reduced levels of nicotine and total leaf alkaloids (wild-type > *nic1* > *nic2* > *nic1 nic2*) (Legg *et al.*, 1969; Legg and Collins, 1971). Although no information is available on the nature of their encoded products, it has been speculated that *Nic1* and *Nic2* likely encode transcriptional regulators capable of globally interacting with a subset of genes encoding components of polyamine and alkaloid biosynthesis (Hibi *et al.*, 1994). Removal of the flower head and several young leaves (i.e., topping) leads to activation of nicotine formation in the roots of decapitated plants (Akehurst, 1981; Hibi *et al.*, 1994). To determine the effects of topping on *NtODC-1* expression in roots, Burley 21 plants were grown in the greenhouse to the bud stage at which point the upper 1/3 of the plant was removed and samples of roots tissues were collected before and at various times post-topping. As shown in Fig 14B, low levels of the *ODC* transcripts were found in roots prior to topping and message abundance increased approximately 2-fold in the roots of topped Burley 21 plants 4 hr after topping. By 24 hr after topping, *ODC* transcript levels return to their initial levels. Low alkaloid mutants of Burley 21 subjected to the same treatment show a much lower level of stimulation of *ODC* transcript accumulation after topping, and the enhanced transcript abundance does not persist beyond 4 hr. By

comparison, transcripts encoding PMT and and a tobacco root-specific β-glucosidase (TBG-1) show patterns of accumulation similar to that observed for ODC transcripts in wild-type plants, but no induction in the low-alkaloid mutant, consistent with previous studies (Hibi *et al.*, 1994; Riechers and Timko, 1999; Wang, 1999).

IV. SAMS

A single recombinant phage is identified as encoding for SAMS. This λ phage contains an approximately 15kB SalI insert. Restriction mapping and PCR analysis indicates that the insert DNA contains primarily the coding and 3'non-coding portions of the SAMS gene. The nucleotide sequences for the gene encoding SAMS can be found at GenBank Accession Nos. AF27243 (full length SAMS cDNA).

V. NADH dehydrogenase

A fragment of the cDNA encoding for NADH dehydrogenase in *N. tabacuum* shows high expression in the roots of mature wild-type HP plants compared to low alkaloid mutant LP plants.

VI. Phosphoribosylanthranilite isomerase (PAI)

The gene encoding for a fragment of phosphoribosylanthranilite isomerase in *N. tabacuum* is a homolog of the *Arabidopsis thaliana* gene encoding PAI, an enzyme involved in tryptophan biosynthesis. This enzyme is involved in the overall formation of aromatic compounds in plants.

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What is claimed is:

1. An isolated DNA molecule comprising the nucleotide sequence of (SEQ. ID. NO. 2), (SEQ. ID. NO. 5), (SEQ. ID. NO. 8), (SEQ. ID. NO. 11), (SEQ. ID. NO. 13), (SEQ. ID. NO. 15), (SEQ. ID. NO. 18), (SEQ. ID. NO. 21), (SEQ. ID. NO. 23), (SEQ. ID. NO. 25) or (SEQ. ID. NO. 26) or comprising a nucleotide sequence encoding the amino acid sequence encoded by (SEQ ID NO. 3), (SEQ. ID. NO. 6), (SEQ ID. NO. 9), (SEQ. ID. NO. 12), (SEQ. ID. NO. 14), (SEQ. ID. NO. 16), (SEQ. ID. NO. 19), (SEQ. ID. NO. 22) OR (SEQ. ID. NO. 24).

2. A vector comprising the isolated DNA molecule of claim 1 operably linked to sequences capable of directing the transcription of a mRNA encoded by said isolated DNA molecule.

3. An isolated DNA molecule comprising a DNA sequence complementary to the nucleotide sequence of claim 1.

4. A vector comprising the isolated DNA molecule of claim 3 operably linked to sequences capable of directing the transcription of a mRNA encoded by said isolated DNA molecule.

5. A cultured transgenic tobacco cell stably transformed with the vector of claim 2.

6. A cultured transgenic tobacco cell stably transformed with the vector of claim 4.

7. A transgenic tobacco plant stably transformed with the vector of claim 2.

8. A transgenic tobacco plant stably transformed with the vector of claim 4.

9. The isolated DNA molecule of claim 1, wherein the isolated DNA molecule comprises the nucleotide sequence of (SEQ ID NO:).

10. A vector comprising the isolated DNA molecule of claim 9 operably linked to sequences capable of directing the transcription of a mRNA encoded by said isolated DNA molecule.

11. An isolated DNA molecule comprising a DNA sequence complementary to the nucleotide sequence of the isolated DNA molecule of claim 9.

12. An isolated DNA sequence comprising about a fifteen to about a twenty-five base pair oligonucleotide sequence identical to any consecutive about fifteen to about twenty-five base pair sequence found in (SEQ. ID. NO. 2), (SEQ. ID. NO. 5), (SEQ. ID. NO. 8), (SEQ. ID. NO. 11), (SEQ. ID. NO. 13), (SEQ. ID. NO. 15), (SEQ. ID. NO. 18), (SEQ. ID. NO. 21), (SEQ. ID. NO. 23), (SEQ. ID. NO. 25) or (SEQ. ID. NO. 26).

13. A cultured transgenic tobacco cell stably transformed with the vector of claim 10.

14.A transgenic tobacco plant stably transformed with the vector of claim 10.

15. A vector comprising a DNA sequence which encodes an antisense mRNA which is complementary to a fragment of a mRNA encoded by the isolated DNA molecule of claim 1, wherein said sequence is operably linked to sequences capable of directing the transcription of said antisense mRNA in tobacco cells and wherein the expression of said antisense mRNA in tobacco cells is sufficient to provide for reduced nicotine content in tobacco cells which are stably transformed with said vector as compared to untransformed tobacco cells.

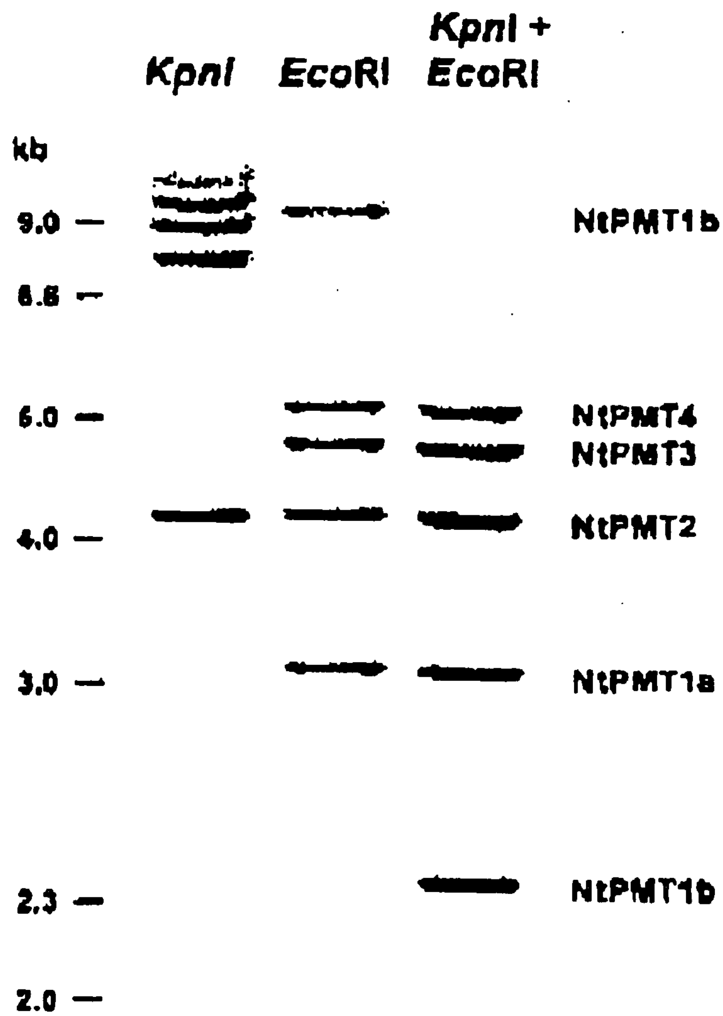
16. A cultured transgenic tobacco cell stably transformed with the vector of claim 15.

17. An isolated and purified protein comprising the amino acid sequence identified in (SEQ ID NO. 3), (SEQ. ID. NO. 6), (SEQ ID. NO. 9), (SEQ. ID. NO. 12), (SEQ. ID. NO. 14), (SEQ. ID. NO. 16), (SEQ. ID. NO. 19), (SEQ. ID. NO. 22) or (SEQ. ID. NO. 24).

18. A method for regulating gene expression in a plant comprising functionally linking an alkaloid gene promoter to a nucleic acid encoding a protein, wherein the promoter comprises a nucleic acid sequence selected from the group consisting of the sequences identified in (SEQ ID NO. 1), (SEQ. ID. NO. 4), (SEQ ID. NO. 7), (SEQ. ID. NO. 10), (SEQ. ID. NO. 17),and (SEQ. ID. NO. 20).

19. The method of claim 18, wherein the nucleic acid encoding a protein encodes a protein involved in the biosynthesis of alkaloids in plants.

20. A plant transformed by the method of claim 18.



KpnI
EcoRI
KpnI + EcoRI
kb
9.0
6.6
6.0
4.0
3.0
2.3
2.0
NtPMT1b
NtPMT4
NtPMT3
NtPMT2
NtPMT1a
NtPMT1b

Figure 1

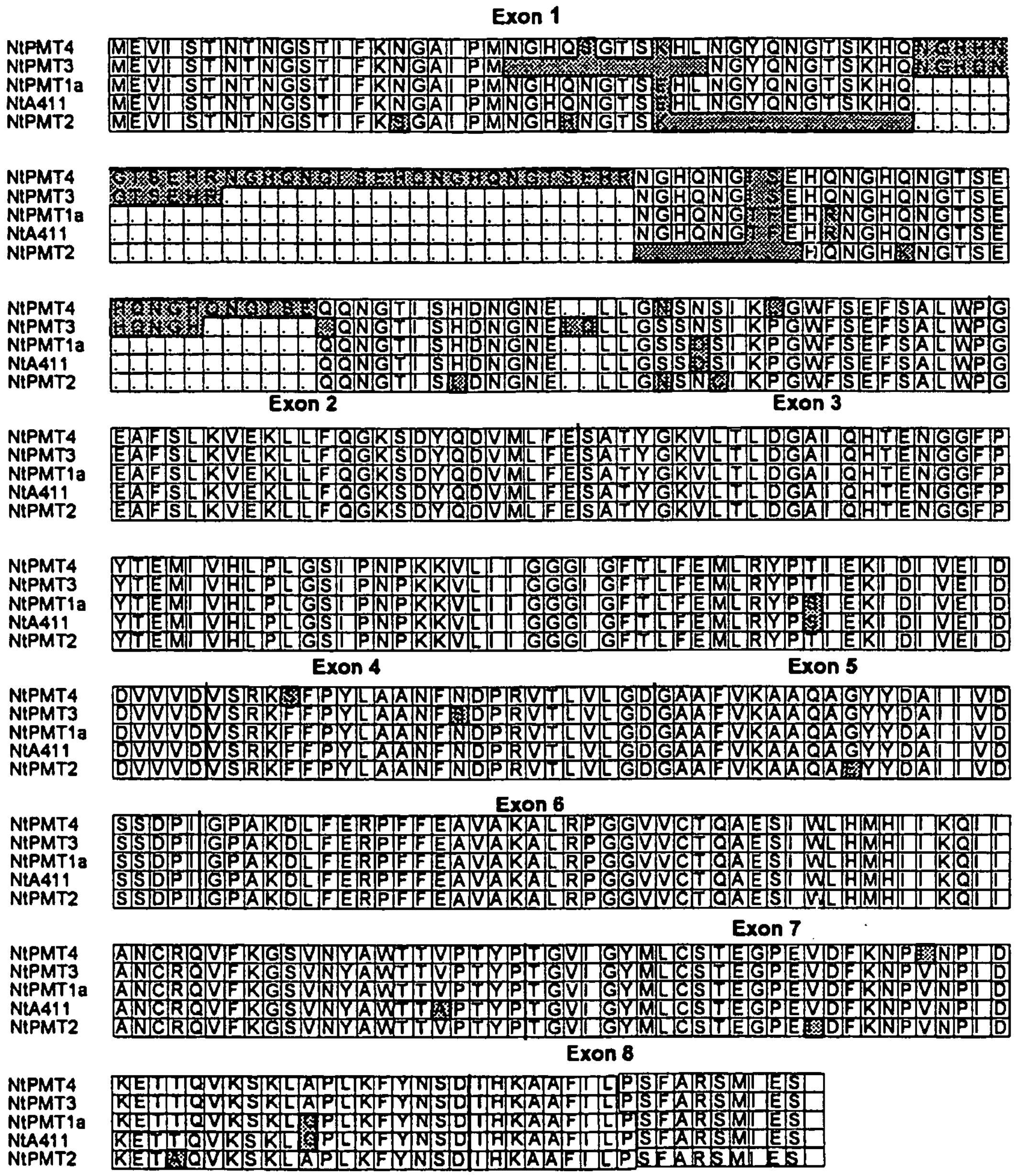

Exon 1
NtPMT4
NtPMT3
NtPMT1a
NtA411
NtPMT2
Exon 2
Exon 3
Exon 4
Exon 5
Exon 6
Exon 7
Exon 8

Figure 2

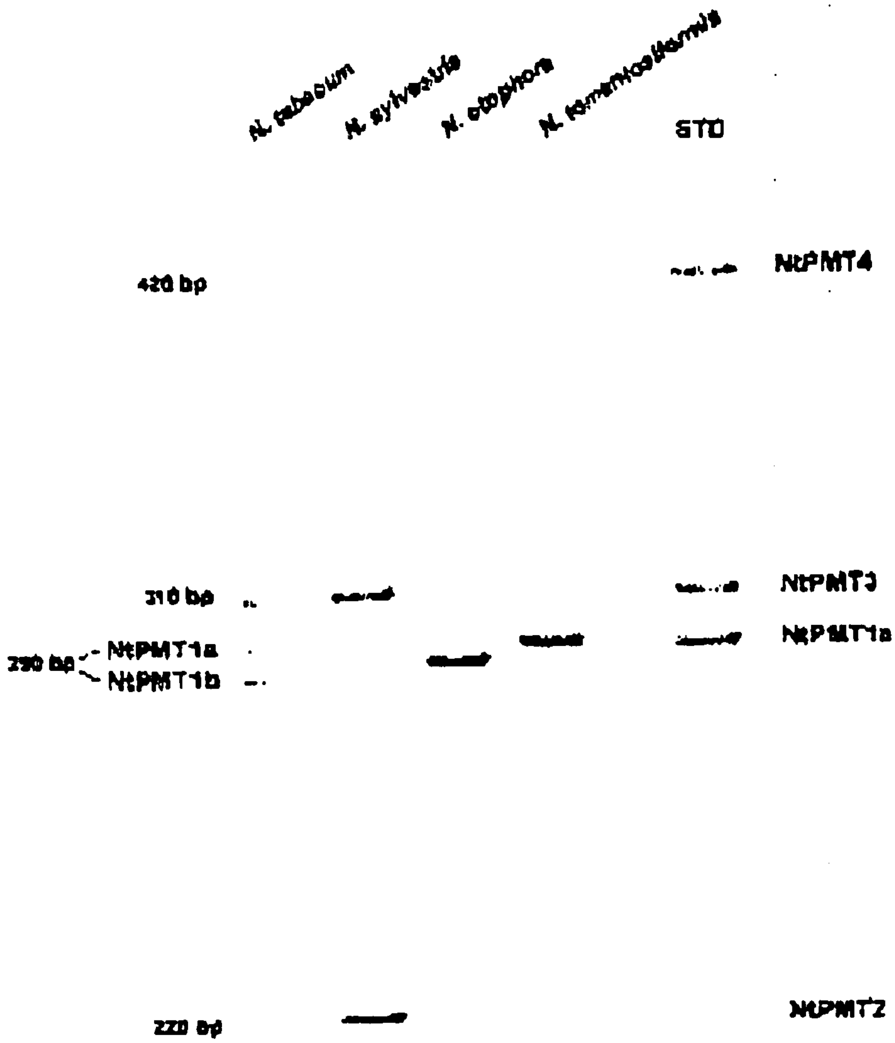

N. tabacum
N. sylvestris
N. otophora
N. tomentosiformis
STD
420 bp
NtPMT4
310 bp
NtPMT3
290 bp
NtPMT1a
NtPMT1b
NtPMT1a
220 bp
NtPMT2

Figure 3

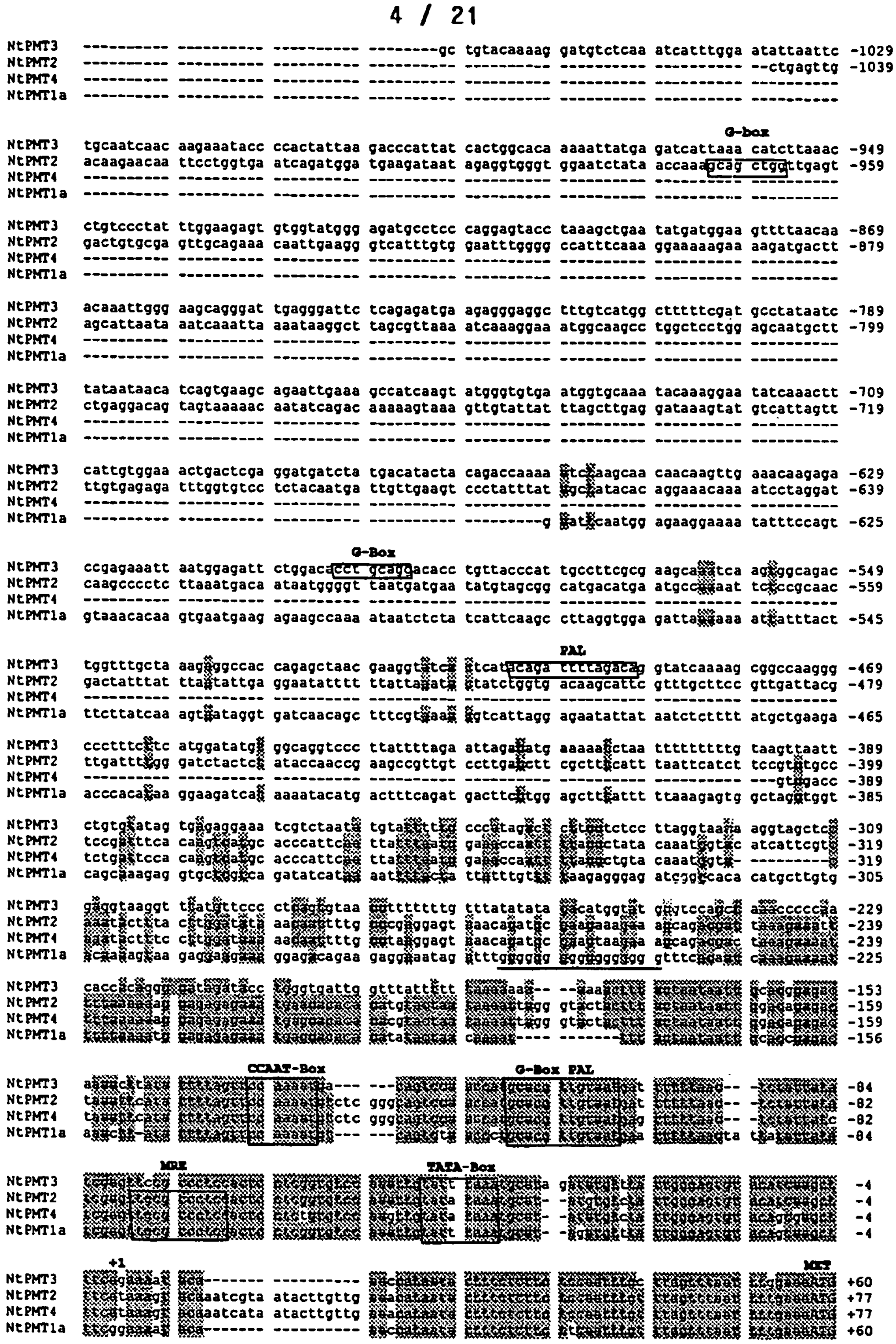

NtPMT3 ---------- ---------- ---------- --------gc tgtacaaaag gatgtctcaa atcatttgga atattaattc -1029
NtPMT2 ---------- ---------- ---------- ---------- ---------- ---------- ---------- --ctgagttg -1039
NtPMT4
NtPMT1a
G-box
NtPMT3 tgcaatcaac aagaaatacc ccactattaa gacccattat cactggcaca aaaattatga gatcattaaa catcttaaac -949
NtPMT2 acaagaacaa ttcctggtga atcagatgga tgaagataat agaggtgggt ggaatctata accaaagcag ctggttgagt -959
NtPMT3 ctgtccctat ttggaagagt gtggtatggg agatgcctcc caggagtacc taaagctgaa tatgatggaa gttttaacaa -869
NtPMT2 gactgtgcga gttgcagaaa caattgaagg gtcatttgtg gaatttgggg ccatttcaaa ggaaaaagaa aagatgactt -879
NtPMT3 acaaattggg aagcagggat tgagggattc tcagagatga agagggaggc tttgtcatgg ctttttcgat gcctataatc -789
NtPMT2 agcattaata aatcaaatta aaataaggct tagcgttaaa atcaaaggaa atggcaagcc tggctcctgg agcaatgctt -799
NtPMT3 tataataaca tcagtgaagc agaattgaaa gccatcaagt atgggtgtga atggtgcaaa tacaaaggaa tatcaaactt -709
NtPMT2 ctgaggacag tagtaaaaac aatatcagac aaaaagtaaa gttgtattat ttagcttgag gataaagtat gtcattagtt -719
G-Box
PAL
CCAAT-Box
G-Box PAL
MRE
TATA-Box
+1
MET
-629 -639 -625 -549 -559 -545 -469 -479 -465 -389 -399 -389 -385 -309 -319 -319 -305 -229 -239 -239 -225 -153 -159 -159 -156 -84 -82 -82 -84 -4 +60 +77 +77 +60

Figure 4

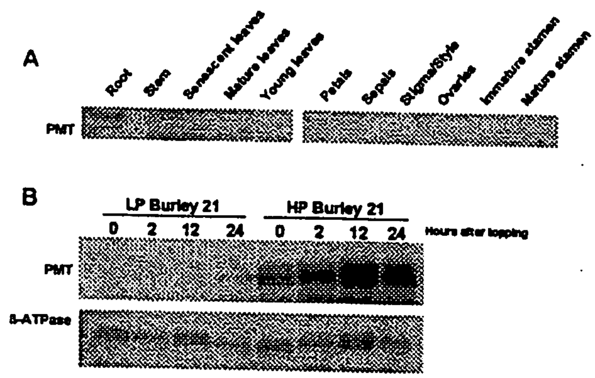

A
Root
Stem
Senescent leaves
Mature leaves
Young leaves
Petals
Sepals
Stigma/Style
Ovaries
Immature stamen
Mature stamen
PMT
B
LP Burley 21
HP Burley 21
0
2
12
24
0
2
12
24
Hours after topping
PMT
ß-ATPase

Figure 5

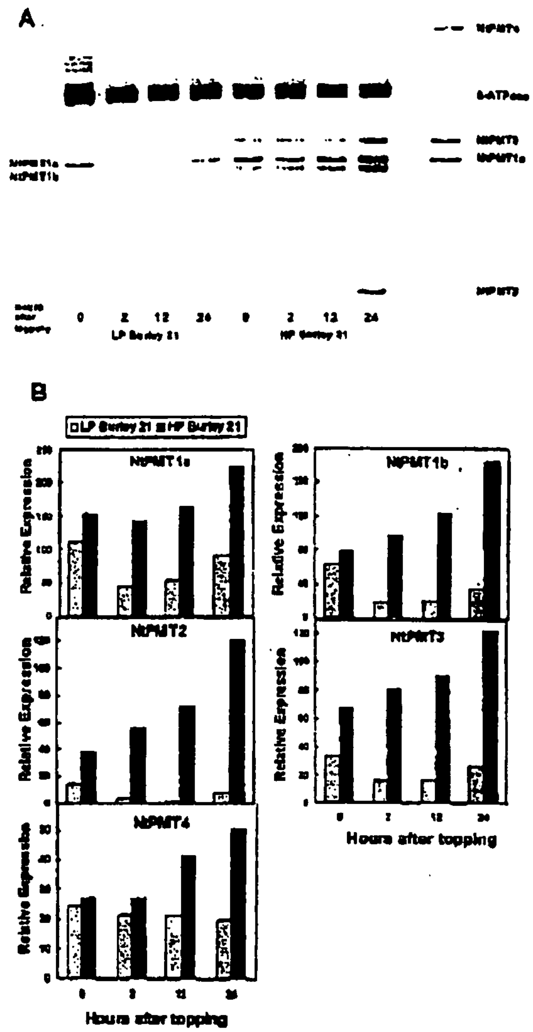

A
B
NtPMT1a
NtPMT1b
NtPMT2
NtPMT3
NtPMT4
Relative Expression
Hours after topping

Figure 6

NtADC1
ttcacgttctcttctcaattcccataaaagaaacccttccgttag
gtttccgtcctatttt--ctcttcttctacgcttc 78
NtADC2
..........................c.........
........................tc...a......c
..t... 80

NtADC1
ctcttctgatatcaatatctgtatggtgtttttcttg
ttcgaatttagatttgttttgcctttaatacctgta
acctta 158
NtADC2
.........a...........................
......................t...........a..
...... 160

NtADC1
taattctctgtttaaaccaaaaacttagcttcttctg
aagtcagggtggggatatttggatcgtgtaagagtgt
gttaga 238
NtADC2 -
....
...............t.....................
..... 239

NtADC1
aggtgattatcttttgattcagttcctttttttgcttc
ttttgaggggggtagccggggcctcggcctcggcgggt
tttaat 318
NtADC2
g....................................
....
. 319

NtADC1
agccccccatctattacaaccattgggcaaaaacatca
ttaaatctgtacaaagcaaacccttaatttagtttaa
ttttct 398
NtADC2
...................t.................
...............a.....................
...... 399

1
M P A L G C C V D A T -
V S P P
NtADC1
gtattctttgattctttaacagaagaagaagagATGC
CGGCCCTAGGTTGTTGCGTAGACGCTACT---
GTTTCCCCTCC 475
NtADC2
......a......t...................ATG.
................T.....T...G..GTT.....
...... 479
1
M * * * * * * * * * A V
* * * *

16 L G Y A F S R D S S
L P A P E F F T S G V P P
T N S A
NtADC1
CTCGGCTATGCCTTCTCTCGGGATAGCTCTCTTCCCG

Figure 7 (a)

CGCCGGAGTTCTTTACCTCCGGCGTACCTCCTACAAA
CTCCG 555
NtADC2
...A.................................
..............G................G.....
...T. 559
17 * S * * * * * * * *
* * * * * * * A * * * * *
* * * *

43 A G S I G S P D L S
S A L Y G V D G W G A P Y
F S V
NtADC1
CCGCCGGTTCCATTGGGTCTCCGGATCTGTCCTCTGC
TTTGTACGGGGTCGATGGGTGGGGAGCTCCTTATTTC
TCCGTT 635
NtADC2
....T.C...................T....G.....
...A.................................
..T... 639
44 * A * * * * * * * *
* * * * * * * * * * * * *
* * *

69 N S N G D I S V R P
H G T D T L P H Q E I D L
L K V V
NtADC1
AACTCTAACGGAGATATCTCCGTCCGACCACATGGTA
CGGACACACTCCCCCACCAGGAAATTGACCTTCTCAA
GGTCGT 715
NtADC2
........T.......................C....
.......T.....T.......................
...... 719
70 * * * * * * * * * *
* * * * * * * * * * * * *
* * * *

96 K K A S D P K N S G
G L G L Q L P L V V R F P
D V L K
NtADC1
GAAAAAGGCCTCCGACCCGAAAAATTCAGGGGGGCTC
GGGCTTCAGCTGCCTCTTGTTGTTCGCTTCCCTGATG
TGCTAA 795
NtADC2
..............................T.....T
....
..T.G. 799
97 * * * * * * * * * *
* * * * * * * * * * * * *
* * * *

123 N R L E S L Q S A F
D L A V H S Q G Y G A H Y
Q G V
NtADC1
AAAACCGGTTGGAATCTCTGCAATCGGCTTTTGATCT
CGCTGTTCATTCCCAGGGCTATGGGGCCCACTACCAA
GGTGTT 875
NtADC2
....

Figure 7 (b)

...G................................
...... 879
124 * * * * * * * * * *
* * * * * * * * * * * * *
* * *

149 Y P V K C N Q D R F
V V E D I V K F G S S F R
F G L E
NtADC1
TATCCCGTGAAATGCAATCAAGACAGGTTCGTGGTGG
AAGATATTGTCAAATTCGGGTCGTCATTCCGGTTCGG
GTTGGA 955
NtADC2
....
.......C...............C............
...... 959
150 * * * * * * * * * *
* * * * * * * * * * P * *
* * * *

176 A G S K P E L L L A
M S C L C R G S A E G L L
V C N G
NtADC1
AGCTGGGTCTAAACCCGAGCTCCTGTTAGCCATGAGC
TGTCTCTGCAGGGGCAGTGCTGAGGGCCTTCTCGTTT
GCAATG 1035
NtADC2
...C................................
..........A.........................
...... 1039

177 * * * * * * * * * *
* * * * * K * * * * * * *
* * * *

203 F K D A E Y I S L A
L V A R K L M L N T V I V
L E Q
NtADC1
GTTTCAAGGACGCTGAGTACATTTCGCTTGCTTTGGT
TGCAAGAAAGCTCATGTTAAACACTGTAATTGTTCTT
GAACAA 1115
NtADC2
....
.................................G...
...... 1119
204 * * * * * * * * * *
* * * * * * * * * * * * *
* * *

229 E E E L D L V I D I
S R K M A V R P V I G L R
A K L R
NtADC1
GAGGAGGAGCTTGACCTTGTGATTGATATAAGCCGTA
AGATGGCTGTTCGGCCCGTAATTGGACTTCGGGCTAA
GCTCAG 1195
NtADC2
.................................A..
...............T....................
...... 1199
230 * * * * * * * * * *
* H * * * * * * * * * * *
* * * *

Figure 7 (c)

256 T K H S G H F G S T S G E K G K F G L T T T Q I V R V
NtADC1
GACCAAGCATTCAGGCCATTTTGGATCCACTTCTGGA
GAAAAAGGTAAGTTTGGGCTTACAACGACCCAAATTG
TTCGTG 1275
NtADC2
....
....
...... 1279
257 *

283 V K K L E E S G M L D C L Q L L H F H I G S Q I P S
NtADC1
TAGTGAAGAAGCTGGAAGAATCCGGAATGCTGGATTG
CCTTCAGTTGCTGCATTTTCACATTGGATCTCAGATC
CCTTCA 1355
NtADC2
.G..........A.......................
T...................................
.....T 1359
284 *

309 T A L L A D G V G E A A Q I Y C E L I R L G A G M K F
NtADC1
ACGGCGTTGCTTGCTGATGGTGTTGGTGAGGCTGCTC
AGATTTATTGTGAATTAATCCGTCTTGGTGCGGGTAT
GAAGTT 1435
NtADC2
....G......A........A...........C....
.................G..........A........
...... 1439
310 * G * * * * * * * * * * * * * * * * V * * * * * * * *

336 I D T G G G L G I D Y D G T K S C D S D V S V G Y G I
NtADC1
CATTGATACTGGAGGTGGGCTCGGAATTGATTATGAT
GGTACTAAATCATGTGATTCAGATGTCTCTGTTGGCT
ATGGCA 1515
NtADC2
........T...........T...............
..............C.....T...............
...... 1519
337 * * I *

Figure 7 (d)

363 Q E Y A S T V V Q A
V Q Y V C D R K G V K H P
V I C
NtADC1
TTCAAGAATACGCCTCCACAGTTGTCCAGGCGGTTCA
ATATGTTTGCGACCGTAAGGGCGTGAAGCACCCAGTG
ATTTGC 1595
NtADC2
..........T......G.......T...........
......A..............T.....A.........
..C... 1599
364 * * * * * * A * * * *
* * * * * * * * * * * * *
* * *

389 S E S G R A I V S H
H S I L I F E A V S A S S
H S C S
NtADC1
AGCGAAAGTGGCAGGGCAATTGTTTCTCATCACTCAA
TTCTGATTTTCGAAGCCGTGTCTGCTTCTAGTCACTC
ATGTTC 1675
NtADC2
....
....
...... 1679
390 * * * * * * * * * * *
* * * * * * * * * * * * *
* * * *

416 S S H L S S G G L Q
S M A E T L N E D A L A D
Y R N L
NtADC1
TTCTTCACATCTGTCTTCTGGTGGCCTCCAATCCATG
GCGGAGACGCTCAATGAAGATGCCCTTGCTGATTACC
GCAATT 1755
NtADC2
....
..............C......................
...... 1759
417 * * * * * * * * * * *
* * * * * * * * * * * * *
* * * *

443 S A A A V R G E Y E
T C V L Y S D Q L K Q R C
V D Q
NtADC1
TATCTGCTGCTGCAGTTCGTGGAGAGTACGAGACGTG
TGTACTTTACTCTGATCAGTTGAAACAGAGATGTGTG
GATCAG 1835
NtADC2
............................T.....A..
....
...... 1839
444 * * * * * * * * * * *
* * * * * * * * * * * * *
* * *

469 F K E G S L G I E H
L A A V D S I C D F V S K
A M G A
NtADC1
TTTAAAGAAGGGTCCTTGGGTATTGAACATCTTGCTG

Figure 7 (e)

CTGTTGATAGCATCTGTGATTTTGTATCAAAGGCTAT
GGGGGC 1915
NtADC2
....
....
...... 1919
470 * * * * * * * * * *
* * * * * * * * * * * * *
* * * *

496 A D P I R T Y H V N
L S I F T S I P D F W A F
G Q L F
NtADC1
TGCTGATCCTATCCGCACTTACCATGTGAATCTGTCA
ATTTTCACTTCAATTCCTGATTTTTGGGCCTTTGGTC
AATTGT 1995
NtADC2
..........G..........................
....
...... 1999
497 * * * V * * * * * *
* * * * * * * * * * * * *
* * * *

523 P I V P I H R L D E
K P A V R G I L S D L T C
D S D
NtADC1
TTCCGATTGTTCCAATACACCGTTTAGATGAAAAGCC
TGCAGTAAGGGGAATATTATCGGACTTGACTTGTGAC
AGTGAT 2075
NtADC2
................T.....C..............
......G...................A.........
...... 2079
524 * * * * * * * * * *
* * * * * * * * * * * * *
* * *

549 G K V D K F I G G E
S S L Q L H E L G S N G D
G G G Y
NtADC1
GGGAAGGTTGATAAGTTCATTGGTGGCGAATCAAGCT
TGCAGCTGCATGAATTGGGAAGTAATGGCGATGGTGG
TGGGTA 2155
NtADC2
....
...C...A.............................
...T.. 2159
550 * * * * * * * * * *
* * * P * * * * * * * * *
* * * *

576 Y L G M F L G G A Y
E E A L G G L H N L F G G
P S V V
NtADC1
TTATCTGGGGATGTTTTTGGGTGGGGCTTATGAGGAG
GCGCTCGGAGGACTCCACAACCTGTTTGGTGGACCAA
GCGTGG 2235
NtADC2
....

Figure 7 (f)

....
.T..C. 2239
577 * * * * * * * * * * *
* * * * * * * * * * * * *
* * * *

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F A M S R S V P G P S C A
D V L
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CATGTCTCGCTCCGTCCCTGGCCCGTCCTGCGCGGAC
GTGCTC 2315
NtADC2
.................................T..
....A.....................T.....T.T.
...... 2319
604 * * * * * * * * * * *
* * * T * * * * * * * * *
* * *

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F E T L K H R A E E F L E
Q E E D
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AGAAGA 2395
NtADC2
....
....
...T.. 2399

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* * D *

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....
..T... 2479
657 * * * * V E * * * *
* V * * * * * * * * * * *
* * S *

683 C C F T A V T A N N
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D S A
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TCTGCT 2555
NtADC2
.....C..........T.C.....A......T.....
......T.............................
...... 2559
684 * R * * * A * D * *
* * * * * * * * * * * * *
* * *

Figure 7(g)

```
   709 T  G  E  D  E  I  W  S  Y  C
T  A  ***

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GAagtgttgtcgtagcatctccagttttagtttgtcg
tcgaag 2635
NtADC2
....................................T
GA......c...........................
....g. 2639
   710 *  *  *  *  *  *  *  *
*  *  *  *  ***


   720
NtADC1
ttgtctgtttttgaataatacccttagttggtgatgt
ttttct
     2678
NtADC2
.............aataata.................
......
     2682
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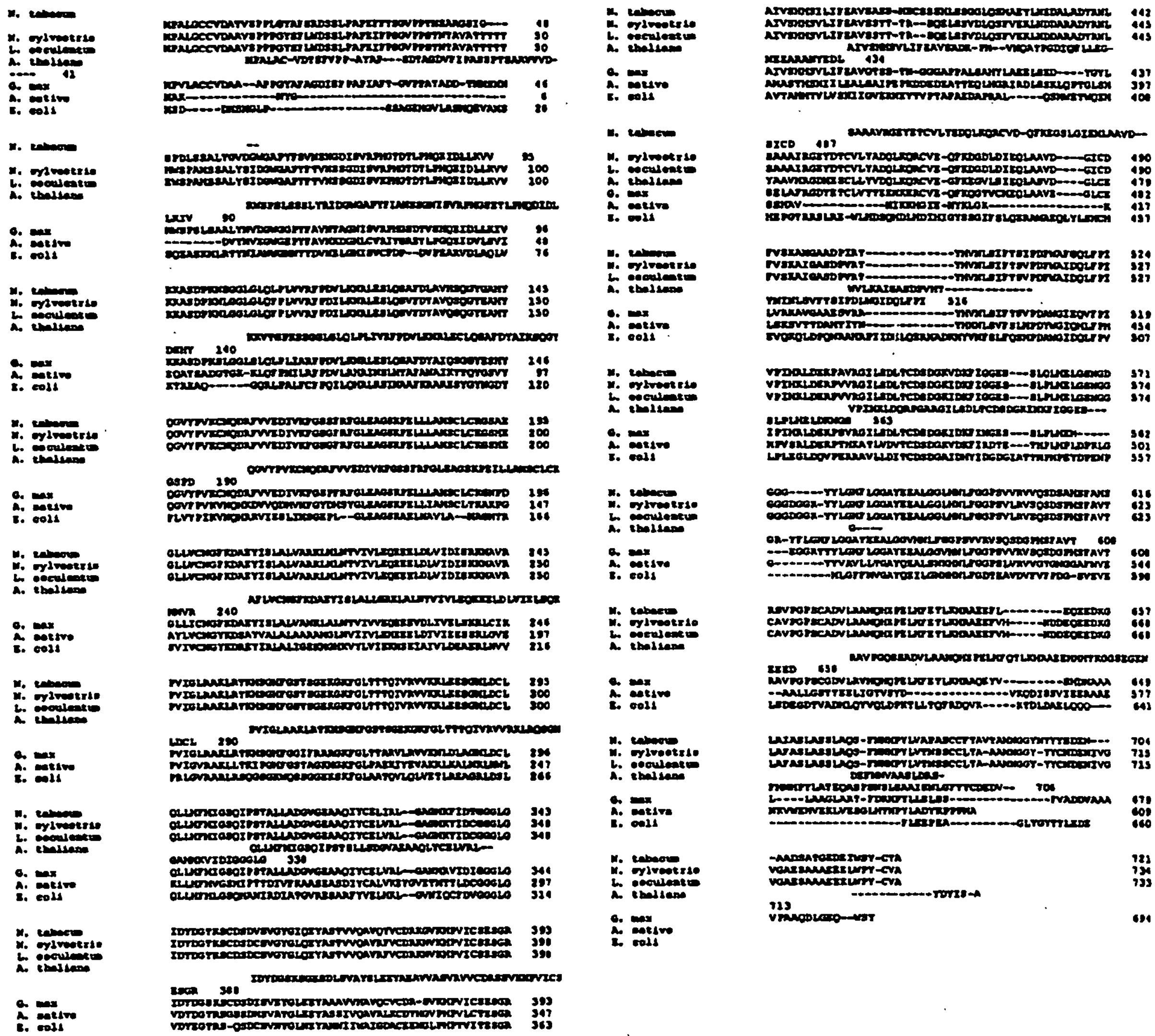
Figure 7(h)

15 / 21



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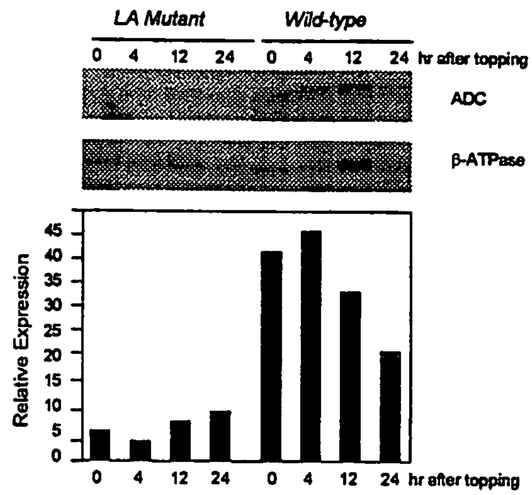

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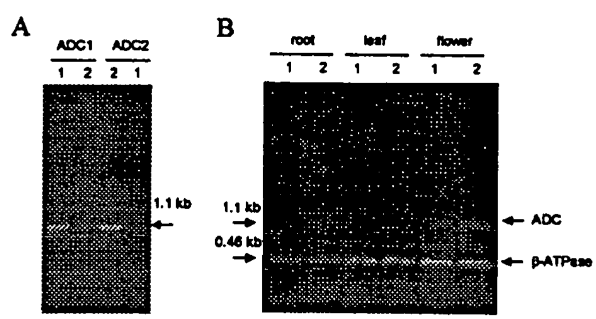

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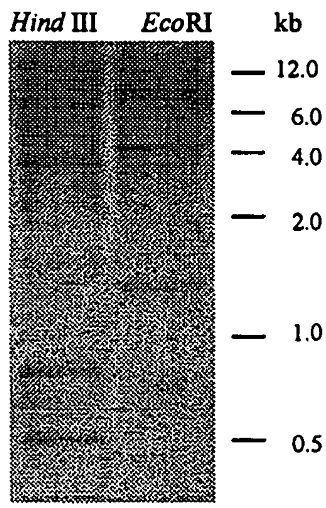

Figure 11

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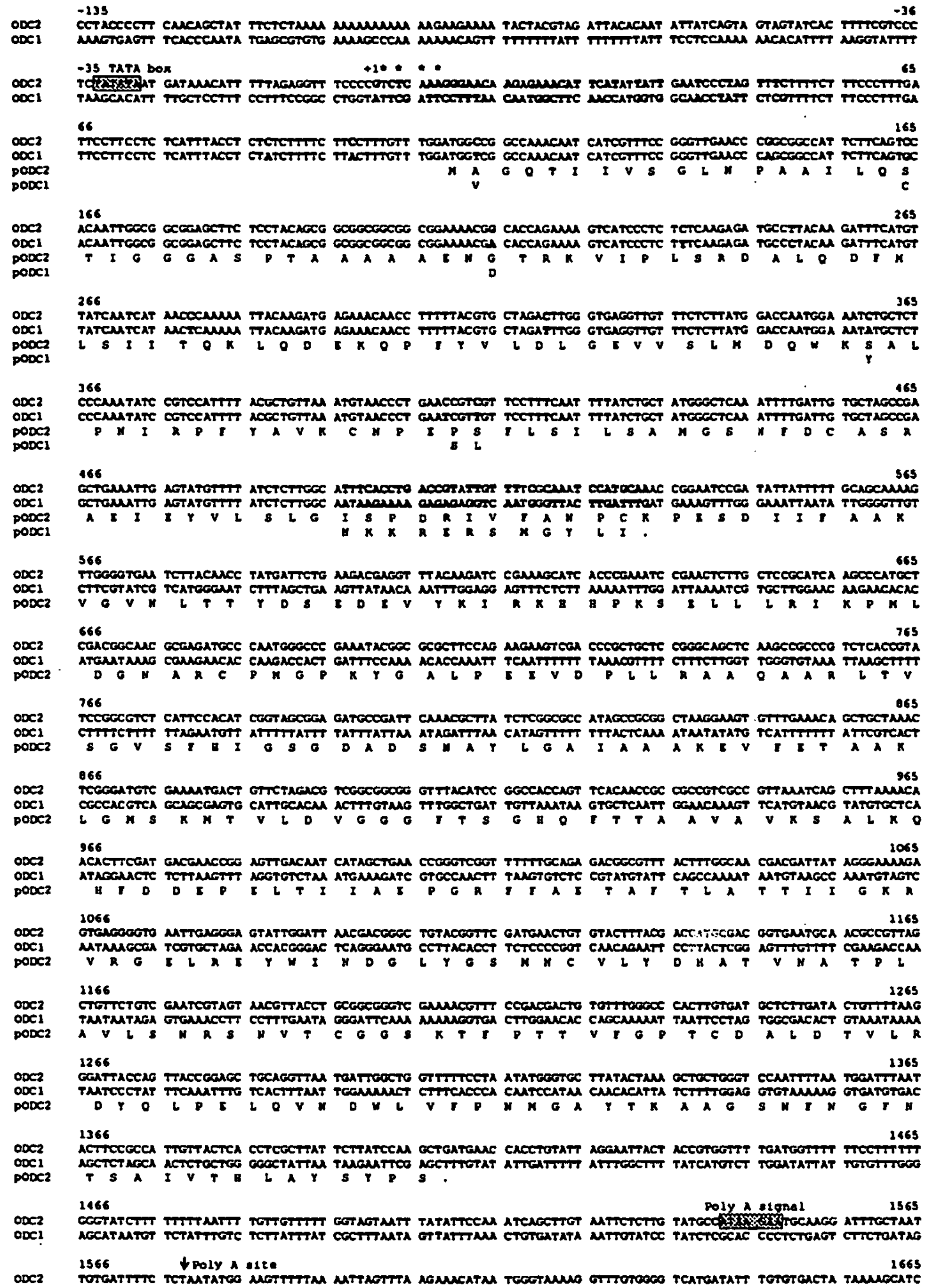

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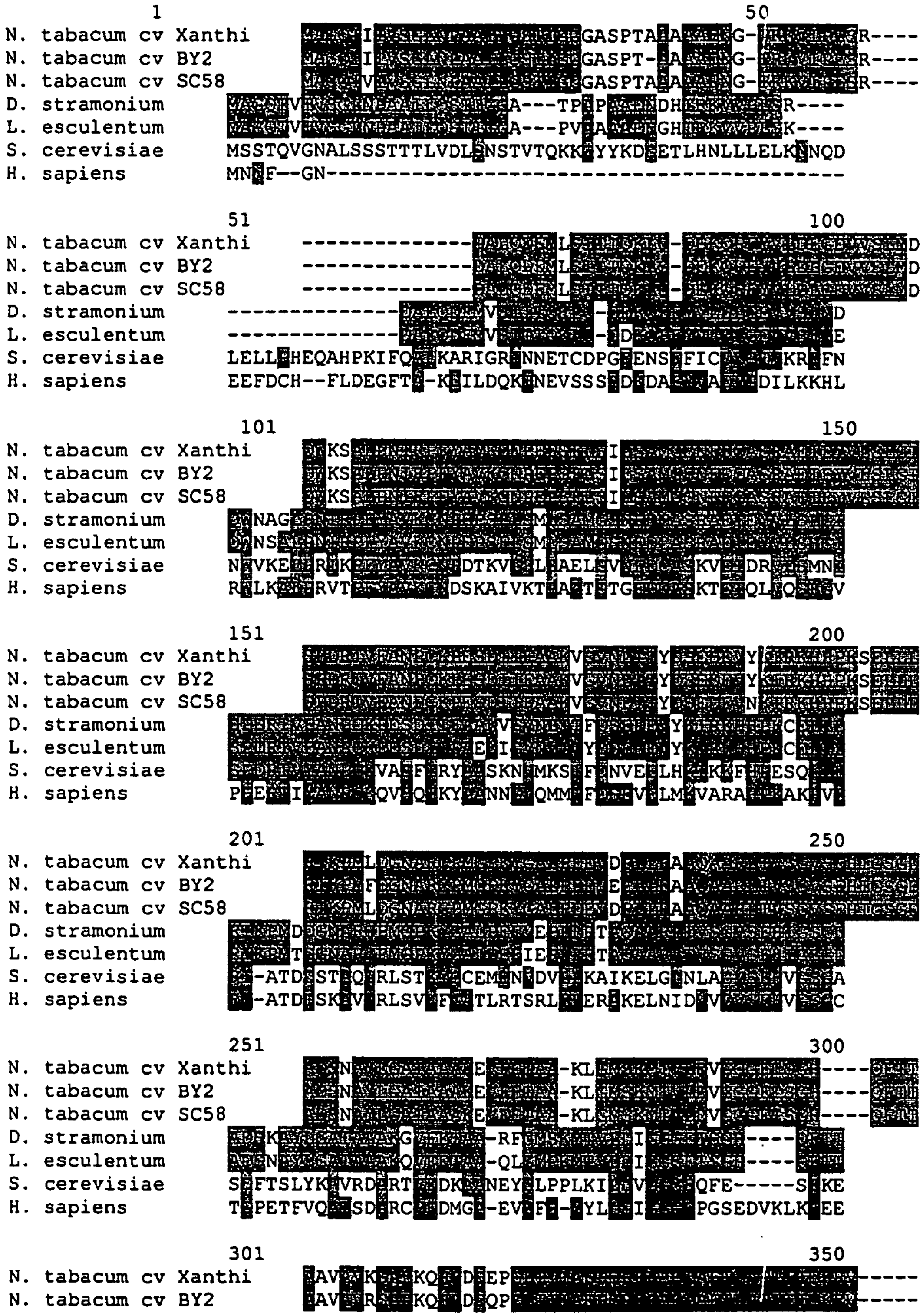
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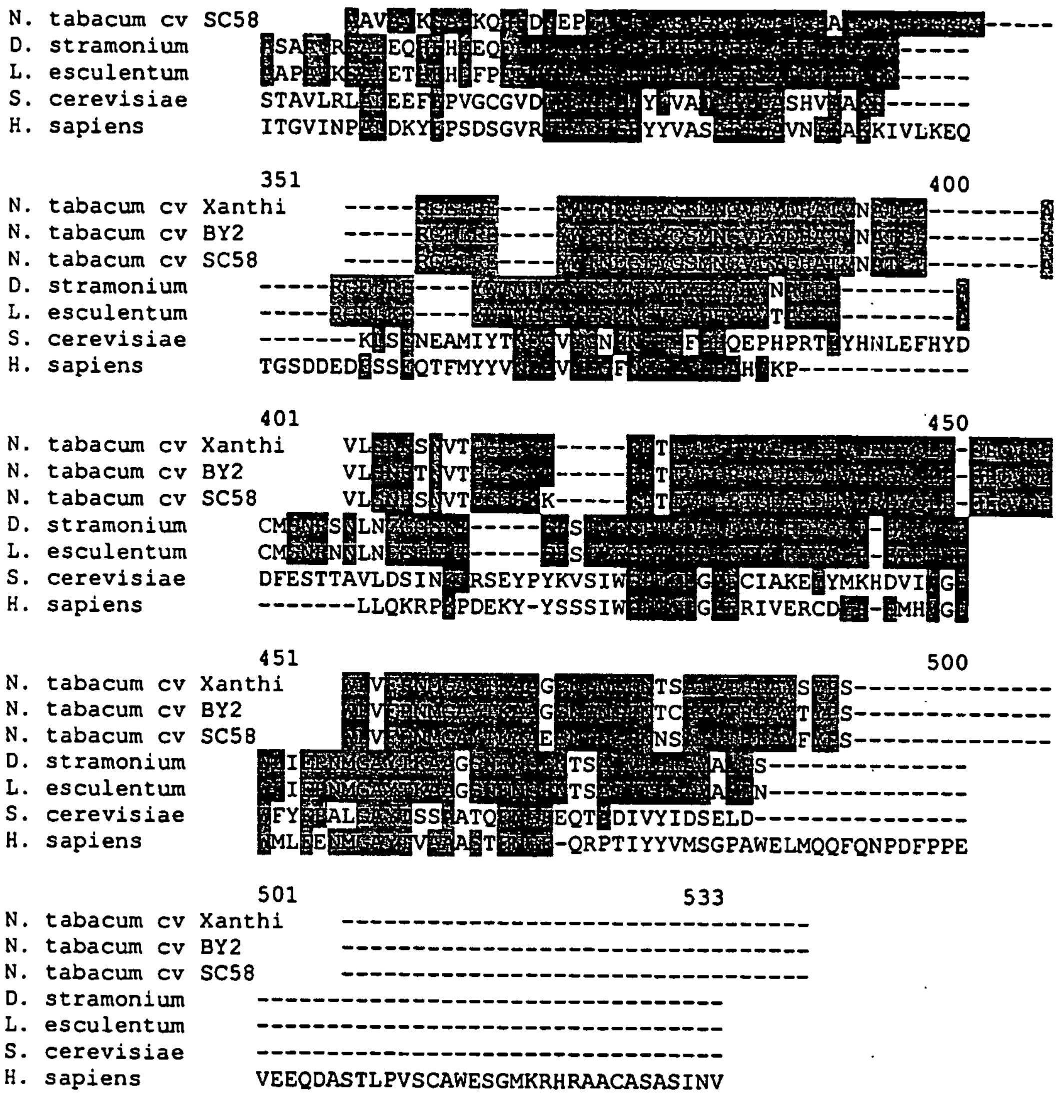
L. esculentum
S. cerevisiae
H. sapiens

Figure 13 (a)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N. tabacum cv SC58
D. stramonium
L. esculentum
S. cerevisiae
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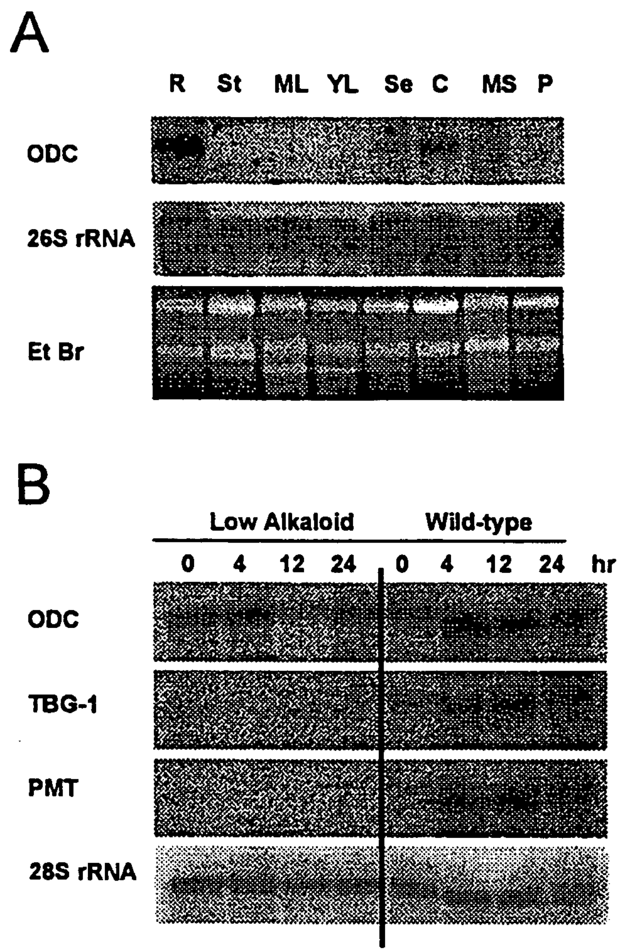
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Figure 14

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Manipulation of Plant Growth and Secondary Metabolism

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Cys Ser Thr Glu Gly Pro Glu Val Asp Phe Lys Asn Pro Val Asn Pro
325 330 335

Ile Asp Lys Glu Thr Thr Gln Val Lys Ser Lys Leu Gly Pro Leu Lys
340 345 350

Phe Tyr Asn Ser Asp Ile His Lys Ala Ala Phe Ile Leu Pro Ser Phe
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Ala Arg Ser Met Ile Glu Ser
370 375

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<213> Plant

<400> 9

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            20                  25                  30

Asn Gly His Gln Asn Gly Thr Ser Glu His Arg Asn Gly His Gln Asn
        35                  40                  45

Gly Ile Ser Glu His Gln Asn Gly His Gln Asn Gly Thr Ser Glu His
    50                  55                  60

Gln Asn Gly His Gln Asn Gly Thr Ile Ser His Asp Asn Gly Asn Glu
65                  70                  75                  80

Leu Gln Leu Leu Gly Ser Ser Asn Ser Ile Lys Pro Gly Trp Phe Ser
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Glu Phe Ser Ala Leu Trp Pro Gly Glu Ala Phe Ser Leu Lys Val Glu
            100                 105                 110

Lys Leu Leu Phe Gln Gly Lys Ser Asp Tyr Gln Asp Val Met Leu Phe
        115                 120                 125

Glu Ser Ala Thr Tyr Gly Lys Val Leu Thr Leu Asp Gly Ala Ile Gln
    130                 135                 140

His Thr Glu Asn Gly Gly Phe Pro Tyr Thr Glu Met Ile Val His Leu
145                 150                 155                 160

Pro Leu Gly Ser Ile Pro Asn Pro Lys Lys Val Leu Ile Ile Gly Gly
                165                 170                 175

Gly Ile Gly Phe Thr Leu Phe Glu Met Leu Arg Tyr Pro Thr Ile Glu
            180                 185                 190

Lys Ile Asp Ile Val Glu Ile Asp Asp Val Val Val Asp Val Ser Arg
        195                 200                 205

Lys Phe Phe Pro Tyr Leu Ala Ala Asn Phe Ser Asp Pro Arg Val Thr
    210                 215                 220

Leu Val Leu Gly Asp Gly Ala Ala Phe Val Lys Ala Ala Gln Ala Gly
225                 230                 235                 240

Tyr Tyr Asp Ala Ile Ile Val Asp Ser Ser Asp Pro Ile Gly Pro Ala
                245                 250                 255

Lys Asp Leu Phe Glu Arg Pro Phe Phe Glu Ala Val Ala Lys Ala Leu
            260                 265                 270
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Arg Pro Gly Gly Val Val Cys Thr Gln Ala Glu Ser Ile Trp Leu His
        275                 280                 285

Met His Ile Ile Lys Gln Ile Ile Ala Asn Cys Arg Gln Val Phe Lys
    290                 295                 300

Gly Ser Val Asn Tyr Ala Trp Thr Thr Val Pro Thr Tyr Pro Thr Gly
305                 310                 315                 320

Val Ile Gly Tyr Met Leu Cys Ser Thr Glu Gly Pro Glu Val Asp Phe
                325                 330                 335

Lys Asn Pro Val Asn Pro Ile Asp Lys Glu Thr Thr Gln Val Lys Ser
            340                 345                 350

Lys Leu Ala Pro Leu Lys Phe Tyr Asn Ser Asp Ile His Lys Ala Ala
        355                 360                 365

Phe Ile Leu Pro Ser Phe Ala Arg Ser Met Ile Glu Ser
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<212> DNA
<213> Plant

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<211> 3001
<212> DNA
<213> Plant

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<211> 419
<212> PRT
<213> Plant

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Gly Ala Ile Pro Met Asn Gly His Gln Ser Gly Thr Ser Lys His Leu
            20   25   30

Asn Gly Tyr Gln Asn Gly Thr Ser Lys His Gln Asn Gly His His Asn
        35   40   45

Gly Thr Ser Glu His Arg Asn Gly His Gln Asn Gly Ile Ser Glu His
    50   55   60

Gln Asn Gly His Gln Asn Gly Thr Ser Glu His Arg Asn Gly His Gln
65   70   75   80

Asn Gly Ile Ser Glu His Gln Asn Gly His Gln Asn Gly Thr Ser Glu
                85   90   95

His Gln Asn Gly His Gln Asn Gly Thr Ser Glu Gln Gln Asn Gly Thr
            100   105   110

Ile Ser His Asp Asn Gly Asn Glu Leu Leu Gly Asn Ser Asn Ser Ile
        115   120   125

Lys Leu Gly Trp Phe Ser Glu Phe Ser Ala Leu Trp Pro Gly Glu Ala
    130   135   140

Phe Ser Leu Lys Val Glu Lys Leu Leu Phe Gln Gly Lys Ser Asp Tyr
145   150   155   160

Gln Asp Val Met Leu Phe Glu Ser Ala Thr Tyr Gly Lys Val Leu Thr
                165   170   175

Leu Asp Gly Ala Ile Gln His Thr Glu Asn Gly Gly Phe Pro Tyr Thr
            180   185   190

Glu Met Ile Val His Leu Pro Leu Gly Ser Ile Pro Asn Pro Lys Lys
        195   200   205

Val Leu Ile Ile Gly Gly Gly Ile Gly Phe Thr Leu Phe Glu Met Leu
    210   215   220

Arg Tyr Pro Thr Ile Glu Lys Ile Asp Ile Val Glu Ile Asp Asp Val

225 230 235 240

Val Val Asp Val Ser Arg Lys Ser Phe Pro Tyr Leu Ala Ala Asn Phe
245 250 255

Asn Asp Pro Arg Val Thr Leu Val Leu Gly Asp Gly Ala Ala Phe Val
260 265 270

Lys Ala Ala Gln Ala Gly Tyr Tyr Asp Ala Ile Ile Val Asp Ser Ser
275 280 285

Asp Pro Ile Gly Pro Ala Lys Asp Leu Phe Glu Arg Pro Phe Phe Glu
290 295 300

Ala Val Ala Lys Ala Leu Arg Pro Gly Gly Val Val Cys Thr Gln Ala
305 310 315 320

Glu Ser Ile Trp Leu His Met His Ile Ile Lys Gln Ile Ile Ala Asn
325 330 335

Cys Arg Gln Val Phe Lys Gly Ser Val Asn Tyr Ala Trp Thr Thr Val
340 345 350

Pro Thr Tyr Pro Thr Gly Val Ile Gly Tyr Met Leu Cys Ser Thr Glu
355 360 365

Gly Pro Glu Val Asp Phe Lys Asn Pro Ile Asn Pro Ile Asp Lys Glu
370 375 380

Thr Thr Gln Val Lys Ser Lys Leu Ala Pro Leu Lys Phe Tyr Asn Ser
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Asp Ile His Lys Ala Ala Phe Ile Leu Pro Ser Phe Ala Arg Ser Met
405 410 415

Ile Glu Ser

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<212> DNA
<213> Plant

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&lt;210&gt; 14
&lt;211&gt; 390
&lt;212&gt; PRT
&lt;213&gt; Plant

&lt;400&gt; 14
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Asp Lys Leu Cys Asp Gln Val Ser Asp Ala Ile Leu Asp Ala Cys Leu
            20                  25                  30

Glu Gln Asp Pro Glu Ser Lys Val Ala Cys Glu Thr Cys Thr Lys Thr
        35                  40                  45

Asn Met Val Met Val Phe Gly Glu Ile Thr Thr Lys Ala Thr Val Asp
    50                  55                  60

Tyr Glu Lys Ile Val Arg Asp Thr Cys Arg Gly Ile Gly Phe Thr Ser
65                  70                  75                  80

Ala Asp Val Gly Leu Asp Ala Asp Asn Cys Lys Val Leu Val Asn Ile
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                85                  90                  95

Glu Gln Gln Ser Pro Asp Ile Ala Gln Gly Val His Gly His Leu Thr
            100                 105                 110

Lys Lys Pro Glu Glu Ile Gly Ala Gly Asp Gln Gly His Met Phe Gly
        115                 120                 125

Tyr Ala Thr Asp Glu Thr Pro Glu Leu Met Pro Leu Thr His Val Trp
    130                 135                 140

Ala Thr Lys Leu Gly Ala Lys Leu Thr Glu Val Arg Lys Asn Lys Thr
145                 150                 155                 160

Cys Pro Trp Leu Arg Pro Asp Gly Lys Thr Gln Val Thr Val Glu Tyr
                165                 170                 175

Lys Asn Asp Asn Gly Ala Met Val Pro Ile Arg Val His Thr Val Leu
            180                 185                 190

Ile Ser Thr Gln His Asp Glu Thr Val Thr Asn Asp Gln Ile Ala Gln
        195                 200                 205

Asp Leu Lys Glu His Val Ile Lys Pro Val Ile Pro Ser Gln Tyr Leu
    210                 215                 220

Asp Glu Asn Thr Ile Phe His Leu Asn Pro Ser Gly Arg Phe Val Ile
225                 230                 235                 240

Gly Gly Pro His Gly Asp Ala Gly Leu Thr Gly Arg Lys Ile Ile Ile
                245                 250                 255

Asp Thr Tyr Gly Gly Trp Gly Ala His Gly Gly Gly Ala Phe Ser Gly
            260                 265                 270

Lys Asp Pro Thr Lys Val Asp Arg Ser Gly Ala Tyr Ile Val Arg Gln
        275                 280                 285

Ala Ala Lys Ser Val Val Ala Ser Gly Leu Ala Arg Arg Cys Ile Val
    290                 295                 300

Gln Val Ser Tyr Ala Ile Gly Val Ala Glu Pro Leu Ser Val Phe Val
305                 310                 315                 320

Asp Thr Tyr Lys Thr Gly Thr Ile Pro Asp Lys Asp Ile Leu Thr Leu
                325                 330                 335

Ile Lys Glu Asn Phe Asp Phe Arg Pro Gly Met Met Ser Ile Asn Leu
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340 345 350

Asp Leu Leu Arg Gly Gly Asn Phe Arg Tyr Gln Lys Thr Ala Ala Tyr
355 360 365

Gly His Phe Gly Arg Asp Asp Pro Asp Phe Ser Trp Glu Thr Val Lys
370 375 380

Val Leu Lys Pro Lys Ala
385 390

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35 40 45

Gln Asp Phe Met Leu Ser Ile Ile Thr Gln Lys Leu Gln Asp Glu Lys
50 55 60

Gln Pro Phe Tyr Val Leu Asp Leu Gly Glu Val Val Ser Leu Met Asp
65 70 75 80

Gln Trp Lys Ser Ala Leu Pro Asn Ile Arg Pro Phe Tyr Ala Val Lys
85 90 95

Cys Asn Pro Glu Pro Ser Phe Leu Ser Ile Leu Ser Ala Met Gly Ser
100 105 110

Asn Phe Asp Cys Ala Ser Arg Ala Glu Ile Glu Tyr Val Leu Ser Leu
115 120 125

Gly Ile Ser Pro Asp Arg Ile Val Phe Ala Asn Pro Cys Lys Pro Glu
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Ser Asp Ile Ile Phe Ala Ala Lys Val Gly Val Asn Leu Thr Thr Tyr
145 150 155 160

Asp Ser Glu Asp Glu Val Tyr Lys Ile Arg Lys His His Pro Lys Ser
165 170 175

Glu Leu Leu Leu Arg Ile Lys Pro Met Leu Asp Gly Asn Ala Arg Cys
180 185 190

Pro Met Gly Pro Lys Tyr Gly Ala Leu Pro Glu Glu Val Asp Pro Leu
195 200 205

Leu Arg Ala Ala Gln Ala Ala Arg Leu Thr Val Ser Gly Val Ser Phe
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His Ile Gly Ser Gly Asp Ala Asp Ser Asn Ala Tyr Leu Gly Ala Ile
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Lys Met Thr Val Leu Asp Val Gly Gly Gly Phe Thr Ser Gly His Gln
            260                 265                 270

Phe Thr Thr Ala Ala Val Ala Val Lys Ser Ala Leu Lys Gln His Phe
        275                 280                 285

Asp Asp Glu Pro Glu Leu Thr Ile Ile Ala Glu Pro Gly Arg Phe Phe
    290                 295                 300

Ala Glu Thr Ala Phe Thr Leu Ala Thr Thr Ile Ile Gly Lys Arg Val
305                 310                 315                 320

Arg Gly Glu Leu Arg Glu Tyr Trp Ile Asn Asp Gly Leu Tyr Gly Ser
                325                 330                 335

Met Asn Cys Val Leu Tyr Asp His Ala Thr Val Asn Ala Thr Pro Leu
            340                 345                 350

Ala Val Leu Ser Asn Arg Ser Asn Val Thr Cys Gly Gly Ser Lys Thr
        355                 360                 365

Phe Pro Thr Thr Val Phe Gly Pro Thr Cys Asp Ala Leu Asp Thr Val
    370                 375                 380

Leu Arg Asp Tyr Gln Leu Pro Glu Leu Gln Val Asn Asp Trp Leu Val
385                 390                 395                 400

Phe Pro Asn Met Gly Ala Tyr Thr Lys Ala Ala Gly Ser Asn Phe Asn
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Gly Phe Asn Thr Ser Ala Ile Val Thr His Leu Ala Tyr Ser Tyr Pro
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35 40 45

Pro Asp Leu Ser Ser Ala Leu Tyr Gly Val Asp Gly Trp Gly Ala Pro
50 55 60

Tyr Phe Ser Val Asn Ser Asn Gly Asp Ile Ser Val Arg Pro His Gly
65 70 75 80

Thr Asp Thr Leu Pro His Gln Glu Ile Asp Leu Leu Lys Val Val Lys
85 90 95

Lys Ala Ser Asp Pro Lys Asn Ser Gly Gly Leu Gly Leu Gln Leu Pro
100 105 110

Leu Val Val Arg Phe Pro Asp Val Leu Lys Asn Arg Leu Glu Ser Leu
115 120 125

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Tyr Gln Gly Val Tyr Pro Val Lys Cys Asn Gln Asp Arg Phe Val Val
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Glu Asp Ile Val Lys Phe Gly Ser Ser Phe Arg Phe Gly Leu Glu Ala
                165                 170                 175

Gly Ser Lys Pro Glu Leu Leu Leu Ala Met Ser Cys Leu Cys Arg Gly
            180                 185                 190

Ser Ala Glu Gly Leu Leu Val Cys Asn Gly Phe Lys Asp Ala Glu Tyr
        195                 200                 205

Ile Ser Leu Ala Leu Val Ala Arg Lys Leu Met Leu Asn Thr Val Ile
    210                 215                 220

Val Leu Glu Gln Glu Glu Glu Leu Asp Leu Val Ile Asp Ile Ser Arg
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Lys Met Ala Val Arg Pro Val Ile Gly Leu Arg Ala Lys Leu Arg Thr
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Lys His Ser Gly His Phe Gly Ser Thr Ser Gly Glu Lys Gly Lys Phe
            260                 265                 270

Gly Leu Thr Thr Thr Gln Ile Val Arg Val Val Lys Lys Leu Glu Glu
        275                 280                 285

Ser Gly Met Leu Asp Cys Leu Gln Leu Leu His Phe His Ile Gly Ser
    290                 295                 300

Gln Ile Pro Ser Thr Ala Leu Leu Ala Asp Gly Val Gly Glu Ala Ala
305                 310                 315                 320

Gln Ile Tyr Cys Glu Leu Ile Arg Leu Gly Ala Gly Met Lys Phe Ile
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Asp Thr Gly Gly Gly Leu Gly Ile Asp Tyr Asp Gly Thr Lys Ser Cys
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Val Val Gln Ala Val Gln Tyr Val Cys Asp Arg Lys Gly Val Lys His
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Glu Asp Ala Leu Ala Asp Tyr Arg Asn Leu Ser Ala Ala Ala Val Arg
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Gly Glu Tyr Glu Thr Cys Val Leu Tyr Ser Asp Gln Leu Lys Gln Arg
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Ala Val Asp Ser Ile Cys Asp Phe Val Ser Lys Ala Met Gly Ala Ala
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His Asn Leu Phe Gly Gly Pro Ser Val Val Arg Val Val Gln Ser Asp
595 600 605

Ser Ala His Ser Phe Ala Met Ser Arg Ser Val Pro Gly Pro Ser Cys
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Ala Asp Val Leu Arg Ala Met Gln His Glu Pro Glu Leu Met Phe Glu
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Thr Leu Lys His Arg Ala Glu Glu Phe Leu Glu Gln Glu Glu Asp Lys
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Gly Leu Ala Ile Ala Ser Leu Ala Ser Ser Leu Ala Gln Ser Phe His
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Asn Met Pro Tyr Leu Val Ala Pro Ala Ser Cys Cys Phe Thr Ala Val
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Phe Ala Ser Gly Val Pro Pro Thr Asn Ser Ala Ala Ala Ser Ile Gly
        35                  40                  45

Ser Pro Asp Leu Ser Ser Ala Leu Tyr Gly Val Asp Gly Trp Gly Ala
    50                  55                  60

Pro Tyr Phe Ser Val Asn Ser Asn Gly Asp Ile Ser Val Arg Pro His
65                  70                  75                  80

Gly Thr Asp Thr Leu Pro His Gln Glu Ile Asp Leu Leu Lys Val Val
                85                  90                  95

Lys Lys Ala Ser Asp Pro Lys Asn Ser Gly Gly Leu Gly Leu Gln Leu
            100                 105                 110

Pro Leu Val Val Arg Phe Pro Asp Val Leu Lys Asn Arg Leu Glu Ser
        115                 120                 125

Leu Gln Ser Ala Phe Asp Leu Ala Val His Ser Gln Gly Tyr Gly Ala
    130                 135                 140

His Tyr Gln Gly Val Tyr Pro Val Lys Cys Asn Gln Asp Arg Phe Val
145                 150                 155                 160

Val Glu Asp Ile Val Lys Phe Gly Ser Pro Phe Arg Phe Gly Leu Glu
                165                 170                 175

Ala Gly Ser Lys Pro Glu Leu Leu Leu Ala Met Ser Cys Leu Cys Lys
            180                 185                 190

Gly Ser Ala Glu Gly Leu Leu Val Cys Asn Gly Phe Lys Asp Ala Glu
        195                 200                 205

Tyr Ile Ser Leu Ala Leu Val Ala Arg Lys Leu Met Leu Asn Thr Val
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Ile Val Leu Glu Gln Glu Glu Glu Leu Asp Leu Val Ile Asp Ile Ser
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225 230 235 240

His Lys Met Ala Val Arg Pro Val Ile Gly Leu Arg Ala Lys Leu Arg
245 250 255

Thr Lys His Ser Gly His Phe Gly Ser Thr Ser Gly Glu Lys Gly Lys
260 265 270

Phe Gly Leu Thr Thr Thr Gln Ile Val Arg Val Val Lys Lys Leu Glu
275 280 285

Glu Ser Gly Met Leu Asp Cys Leu Gln Leu Leu His Phe His Ile Gly
290 295 300

Ser Gln Ile Pro Ser Thr Gly Leu Leu Ala Asp Gly Val Gly Glu Ala
305 310 315 320

Ala Gln Ile Tyr Cys Glu Leu Val Arg Leu Gly Ala Gly Met Lys Phe
325 330 335

Ile Asp Ile Gly Gly Gly Leu Gly Ile Asp Tyr Asp Gly Thr Lys Ser
340 345 350

Cys Asp Ser Asp Val Ser Val Gly Tyr Gly Ile Gln Glu Tyr Ala Ser
355 360 365

Ala Val Val Gln Ala Val Gln Tyr Val Cys Asp Arg Lys Gly Val Lys
370 375 380

His Pro Val Ile Cys Ser Glu Ser Gly Arg Ala Ile Val Ser His His
385 390 395 400

Ser Ile Leu Ile Phe Glu Ala Val Ser Ala Ser Ser His Ser Cys Ser
405 410 415

Ser Ser His Leu Ser Ser Gly Gly Leu Gln Ser Met Ala Glu Thr Leu
420 425 430

Asn Glu Asp Ala Leu Ala Asp Tyr Arg Asn Leu Ser Ala Ala Ala Val
435 440 445

Arg Gly Glu Tyr Glu Thr Cys Val Leu Tyr Ser Asp Gln Leu Lys Gln
450 455 460

Arg Cys Val Asp Gln Phe Lys Glu Gly Ser Leu Gly Ile Glu His Leu
465 470 475 480

Ala Ala Val Asp Ser Ile Cys Asp Phe Val Ser Lys Ala Met Gly Ala

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Ala Asp Pro Val Arg Thr Tyr His Val Asn Leu Ser Ile Phe Thr Ser
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Ile Pro Asp Phe Trp Ala Phe Gly Gln Leu Phe Pro Ile Val Pro Ile
        515                 520                 525

His Arg Leu Asp Glu Lys Pro Ala Val Arg Gly Ile Leu Ser Asp Leu
    530                 535                 540

Thr Cys Asp Ser Asp Gly Lys Val Asp Lys Phe Ile Gly Gly Glu Ser
545                 550                 555                     560

Ser Leu Pro Leu His Glu Leu Gly Ser Asn Gly Asp Gly Gly Gly Tyr
                565                 570                 575

Tyr Leu Gly Met Phe Leu Gly Gly Ala Tyr Glu Glu Ala Leu Gly Gly
            580                 585                 590

Leu His Asn Leu Phe Gly Gly Pro Ser Val Val Arg Val Val Gln Ser
        595                 600                 605

Asp Ser Ala His Ser Phe Ala Met Thr Arg Ser Val Pro Gly Pro Ser
    610                 615                 620

Cys Ala Asp Val Leu Arg Ala Met Gln His Glu Pro Glu Leu Met Phe
625                 630                 635                     640

Glu Thr Leu Lys His Arg Ala Glu Glu Phe Leu Glu Gln Glu Asp Asp
                645                 650                 655

Lys Gly Leu Ala Val Glu Ser Leu Ala Ser Ser Val Ala Gln Ser Phe
            660                 665                 670

His Asn Met Pro Tyr Leu Val Ala Pro Ser Ser Cys Arg Phe Thr Ala
        675                 680                 685

Ala Thr Asp Asn Asn Gly Gly Tyr Asn Tyr Tyr Tyr Ser Asp Glu Asn
    690                 695                 700

Ala Ala Asp Ser Ala Thr Gly Glu Asp Glu Ile Trp Ser Tyr Cys Thr
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<211> 720
<212> PRT
<213> Plant

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            20   25   30

Thr Ser Gly Val Pro Pro Thr Asn Ser Ala Ala Gly Ser Ile Gly Ser
        35   40   45

Pro Asp Leu Ser Ser Ala Leu Tyr Gly Val Asp Gly Trp Gly Ala Pro
    50   55   60

Tyr Phe Ser Val Asn Ser Asn Gly Asp Ile Ser Val Arg Pro His Gly
65   70   75   80

Thr Asp Thr Leu Pro His Gln Glu Ile Asp Leu Leu Lys Val Val Lys
                85   90   95

Lys Ala Ser Asp Pro Lys Asn Ser Gly Gly Leu Gly Leu Gln Leu Pro
            100   105   110

Leu Val Val Arg Phe Pro Asp Val Leu Lys Asn Arg Leu Glu Ser Leu
        115   120   125

Gln Ser Ala Phe Asp Leu Ala Val His Ser Gln Gly Tyr Gly Ala His
    130   135   140

Tyr Gln Gly Val Tyr Pro Val Lys Cys Asn Gln Asp Arg Phe Val Val
145   150   155   160

Glu Asp Ile Val Lys Phe Gly Ser Ser Phe Arg Phe Gly Leu Glu Ala
                165   170   175

Gly Ser Lys Pro Glu Leu Leu Leu Ala Met Ser Cys Leu Cys Arg Gly
            180   185   190

Ser Ala Glu Gly Leu Leu Val Cys Asn Gly Phe Lys Asp Ala Glu Tyr
        195   200   205

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Ile Ser Leu Ala Leu Val Ala Arg Lys Leu Met Leu Asn Thr Val Ile
    210                 215                 220

Val Leu Glu Gln Glu Glu Glu Leu Asp Leu Val Ile Asp Ile Ser Arg
225                 230                 235                 240

Lys Met Ala Val Arg Pro Val Ile Gly Leu Arg Ala Lys Leu Arg Thr
                245                 250                 255

Lys His Ser Gly His Phe Gly Ser Thr Ser Gly Glu Lys Gly Lys Phe
            260                 265                 270

Gly Leu Thr Thr Thr Gln Ile Val Arg Val Val Lys Lys Leu Glu Glu
        275                 280                 285

Ser Gly Met Leu Asp Cys Leu Gln Leu Leu His Phe His Ile Gly Ser
    290                 295                 300

Gln Ile Pro Ser Thr Ala Leu Leu Ala Asp Gly Val Gly Glu Ala Ala
305                 310                 315                 320

Gln Ile Tyr Cys Glu Leu Ile Arg Leu Gly Ala Gly Met Lys Phe Ile
                325                 330                 335

Asp Thr Gly Gly Gly Leu Gly Ile Asp Tyr Asp Gly Thr Lys Ser Cys
            340                 345                 350

Asp Ser Asp Val Ser Val Gly Tyr Gly Ile Gln Glu Tyr Ala Ser Thr
        355                 360                 365

Val Val Gln Ala Val Gln Tyr Val Cys Asp Arg Lys Gly Val Lys His
    370                 375                 380

Pro Val Ile Cys Ser Glu Ser Gly Arg Ala Ile Val Ser His His Ser
385                 390                 395                 400

Ile Leu Ile Phe Glu Ala Val Ser Ala Ser Ser His Ser Cys Ser Ser
                405                 410                 415

Ser His Leu Ser Ser Gly Gly Leu Gln Ser Met Ala Glu Thr Leu Asn
            420                 425                 430

Glu Asp Ala Leu Ala Asp Tyr Arg Asn Leu Ser Ala Ala Ala Val Arg
        435                 440                 445

Gly Glu Tyr Glu Thr Cys Val Leu Tyr Ser Asp Gln Leu Lys Gln Arg
    450                 455                 460
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Cys Val Asp Gln Phe Lys Glu Gly Ser Leu Gly Ile Glu His Leu Ala
465                 470                 475                 480

Ala Val Asp Ser Ile Cys Asp Phe Val Ser Lys Ala Met Gly Ala Ala
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Asp Pro Ile Arg Thr Tyr His Val Asn Leu Ser Ile Phe Thr Ser Ile
            500                 505                 510

Pro Asp Phe Trp Ala Phe Gly Gln Leu Phe Pro Ile Val Pro Ile His
        515                 520                 525

Arg Leu Asp Glu Lys Pro Ala Val Arg Gly Ile Leu Ser Asp Leu Thr
    530                 535                 540

Cys Asp Ser Asp Gly Lys Val Asp Lys Phe Ile Gly Gly Glu Ser Ser
545                 550                 555                 560

Leu Gln Leu His Glu Leu Gly Ser Asn Gly Asp Gly Gly Gly Tyr Tyr
                565                 570                 575

Leu Gly Met Phe Leu Gly Gly Ala Tyr Glu Glu Ala Leu Gly Gly Leu
            580                 585                 590

His Asn Leu Phe Gly Gly Pro Ser Val Val Arg Val Val Gln Ser Asp
        595                 600                 605

Ser Ala His Ser Phe Ala Met Ser Arg Ser Val Pro Gly Pro Ser Cys
    610                 615                 620

Ala Asp Val Leu Arg Ala Met Gln His Glu Pro Glu Leu Met Phe Glu
625                 630                 635                 640

Thr Leu Lys His Arg Ala Glu Glu Phe Leu Glu Gln Glu Glu Asp Lys
                645                 650                 655

Gly Leu Ala Ile Ala Ser Leu Ala Ser Ser Leu Ala Gln Ser Phe His
            660                 665                 670

Asn Met Pro Tyr Leu Val Ala Pro Ala Ser Cys Cys Phe Thr Ala Val
        675                 680                 685

Thr Ala Asn Asn Gly Gly Tyr Asn Tyr Tyr Tyr Ser Asp Glu Asn Ala
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Ala Asp Ser Ala Thr Gly Glu Asp Glu Ile Trp Ser Tyr Cys Thr Ala
705                 710                 715                 720
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<213> Plant

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catgaagtac gtatattaca cgtttttccc tgaccaaagc ttaatcgaat tctgcagccc 600
gggggatcng gnattctaga gcggcgccac gcggtggagc tccaatcgcc taaatgancn 660
ataaaatcac tggccgtcgt ttanacgncn ggacgggaaa cctgggtacc aacttaatcg 720
cctgnagcna tccccttcnc agcggngtan acgaaaggcc gncgattgcc tccanattgc 780
cacnggatgg aanggacncc gtncgganga acngggggnn ggggtaccn 829

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/12450

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A01H 5/00; C07H 21/04; C12N 5/14, 15/29, 15/52, 15/82
US CL :435/320.1, 414, 419; 536/23.2, 23.6, 24.5; 800/278, 317.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 414, 419; 536/23.2, 23.6, 24.5; 800/278, 317.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	HASHIMOTO et al. Intraspecific Variability of the Tandem Repeats in Nicotiana Putrescine N-methyltransferase. Plant Molecular Biology. 1998, Vol. 37, pages 25-37, especially Figure 3.	12 ---- 15,16
X --- Y	HIBI et al. Gene Expression in Tobacco Low-Nicotine Mutants. The Plant Cell. May 1994, Vol. 6, pages 723-735, especially Figure 3.	12 ---- 15,16
X --- Y	IZHAKI et al. A Petunia cDNA Encoding S-Adenosylmethionine Synthetase. Plant Physiology. 1995, Vol. 108, pages 841-842, see entire article.	12 ---- 15,16

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 17 AUGUST 2000	Date of mailing of the international search report 04 OCT 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer AMY NELSON Terry J Dey for Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/12450

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	LAMATTINA et al. RNA Editing of the Transcript Coding for Subunit 4 of NADH Dehydrogenase in Wheat Mitochondria: Uneven Distribution of the Editing Sites Among the Four Exons. Nucleic Acids Research 1991, Vol. 19, No. 12, pages 3275-3282, especially Figure 4.	12 ---- 15,16
X --- Y	LI et al. Arabidopsis Phosphoribosylanthranilate Isomerase: Molecular Genetic Analysis of Triplicate Tryptophan Pathway Genes. The Plant Cell. April 1995, Vol. 7, pages 447-461, especially Figure 3, page 459.	12,15 ---- 16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/12450

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [X] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-15,18-20

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest [X] The additional search fees were accompanied by the applicant's protest.
[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/12450

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

STN, AGRICOLA, CAPLUS, BIOSIS, EMBASE, USPAT
search terms: putrescine methyltransferase, adenosylmethionine synthetase, ornithine decarboxylase, arginine decarboxylase, NADH dehydrogenase, phosphoribosylanthranilate isomerase, DNA, cDNA, gene, nucleic

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-16, drawn to coding DNA, vector, host cell, transgenic plant.
Group II, claim(s) 17, drawn to protein.
Group III, claim(s) 18-20, drawn to transformation method and transgenic plant with promoter DNA.

The inventions listed as Groups I, II, and III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The coding DNA of Group I, *e.g.* Claim 12, is disclosed in the prior art publication of Hashimoto *et al.* (Plant Mol. Biol. 37: 25-37, 1998; see Fig. 3b). Therefore, there is no special technical feature which links the coding DNA of Group I with the protein of Group II.

Furthermore, there is no special technical feature under PCT Rule 13.2 which links the coding DNA of Group I and the transformation method and transgenic plant with the promoter DNA of Group III. Therefore, the inventions of Groups I, II, and III do no relate to a single inventive concept under PCT Rule 13.1.